Determining the antibacterial potential of a medium-chain fatty acid, caprylic acid, against multidrug-resistant *Salmonella* enterica *enterica* serovar Heidelberg in broiler chickens

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Abstract

Salmonella is the leading bacterial cause of foodborne illness in the United States of America. Among the various foodborne infections, salmonellosis ranks first in terms of hospitalizations and death. More than 50% of foodborne salmonellosis is attributed to the consumption of contaminated poultry products. *Salmonella* colonizes the chicken cecum and gets excreted, leading to the contamination of the farm environment and poultry carcass during processing. Among the >2500 serovars of *Salmonella* causing human infections, 7% are associated with foodborne outbreaks through poultry. *Salmonella* enterica serovar Heidelberg (SH) has emerged through many foodborne outbreaks and found to be resistant to various commonly used and clinically relevant antibiotics. Multidrug-resistant (MDR) SH is one of the commonly isolated *Salmonella* from chicken carcasses and is associated with foodborne outbreaks through chicken products.

Due to the federal initiatives to curb the use of antibiotic resistance development in animal agriculture, alternative antimicrobial strategies that control all *Salmonella*, including SH, is considered the urgent need of the poultry food industry. Since the pathogen is resistant to clinically important antibiotics, strategies are required to control them on farms and processing. Medium-chain fatty acids (MCFAs) could be an effective alternative to antibiotics approach as MCFAs have broad-spectrum antimicrobial activity. Among the MCFA's, caprylic acid (CA) has been reported to have antibacterial potential against *Salmonella*. In this thesis, two questions were asked: Could a long-term supplementation of CA through feed control cecal colonization of MDR SH in broiler

chickens (preharvest strategy), and could CA be effective against MDR SH on chicken drumsticks when applied in scalding water (processing aid).

At first, we investigated the efficacy of CA in reducing MDR SH colonization in the cecum of 5-week old broiler chickens. Two independent studies were conducted. In each experiment, day-old Ross 708 broiler chicks were randomly allocated to four different groups (3 chicks/group; two studies). The four groups included in the study were: Negative control (NC), Positive Control (PC), Antibiotic group (AB), and CA group (CA). The birds in NC and PC were fed with a standard basal diet, whereas the broilers in the AB group received a standard diet containing 50g bacitracin methylene disalicylate (BMD)/ ton of feed for 5 weeks. The CA (1% w/w) was supplemented through the feed to the broilers in the CA group for 5 weeks. All birds except those in the NC group were challenged with 3.69 $\log_{10}$ CFU MDR SH (2014 Tennessee correctional facility outbreak strain) by crop gavage. Birds were euthanized 7-days after MDR SH inoculation by CO_2 asphyxiation. Cecal samples were collected, and the cecal colonization of *Salmonella* was determined after plating the homogenates onto xylose lysine deoxycholate agar (XLD) plates. The bacterial counts were transformed to log values, and ANOVA was used for statistical analysis. The BMD supplementation resulted in 3.4 and 4.0 $\log_{10}$ CFU/g reduction of MDR SH in studies 1 and 2, respectively. The CA supplementation also resulted in a comparable reduction in cecal colonization of MDR SH as that of BMD. A reduction of 3.2 and 4.0 $\log_{10}$ CFU/g was observed in studies 1 and 2, respectively, in the CA group compared to birds in PC. Therefore, CA could be used as an effective control strategy against MDR SH colonization in 5-week old broilers. This results corroborate with other studies employing CA to control another major *Salmonella* serovar in broiler chickens, *S.* Enteritidis.

In the second study, we determined the antimicrobial efficacy of CA against MDR SH on chicken drumsticks in simulated soft scalding conditions. Chicken drumsticks were spot inoculated with MDR SH [either lower (~3.0 $\log_{10}$ CFU/g) or higher (~5.0 $\log_{10}$ CFU/g) inoculum] and immersed in scalding water containing treatments for 2 min at 54^{o}C (USDA-recommended time-temperature combination for soft scalding). The antimicrobial treatments included in the study were 0.5% CA, 1% CA, 0.05% peracetic acid (PAA), 0.5% CA + 0.05% PAA and 1.0% CA + 0.05% PAA. Samples inoculated with or without MDR SH and immersed in scalding water containing neither of the antimicrobial treatments served as the PC and NC groups, respectively. Immediately after scalding, the drumsticks were homogenized in phosphate-buffered saline (PBS), and surviving MDR SH populations were recovered on XLD agar plates (n=6). Similarly, MDR SH populations that survived in scalding water was also determined after surface plating (n=6). Additionally, the efficacy of the scalding treatments against MDR SH survival on drumsticks for a storage period of 48 h at 4^{o}C was determined. Furthermore, the effect of these treatments on the surface color of the drumsticks was also evaluated. The antimicrobial treatments resulted in a significant reduction of MDR SH on drumsticks. For the lower inoculum, 0.5% CA, 1% CA, 0.05% PAA, 0.5% CA + 0.05% PAA and 1.0% CA + 0.05% PAA resulted in 0.7, 1.0, 2.5, 1.4 and 1.5 $\log_{10}$ CFU/g reduction of MDR SH on drumsticks ($P<0.05$). The same treatments resulted in 0.9, 1.3, 2.5, 2.2, and 2.6 $\log_{10}$ CFU/g reduction of MDR SH when the drumsticks were contaminated with the higher inoculum level ($P<0.05$). Moreover, the antimicrobial treatments completely inactivated MDR SH in scalding water to undetectable levels, whereas 2.0 to 4.0 $\log_{10}$ CFU/mL MDR SH survived in the PC group ($P<0.05$). Also, the scalding treatments were effective in

inhibiting MDR SH on the drumsticks compared to the respective controls during a storage period of 48 h at 4^{o}C ($P<0.05$), although the magnitude of reduction remained the same as observed during the scalding treatment. Additionally, none of the treatments affected the color of the drumsticks ($P>0.05$). The results indicated that CA could be used as an effective intervention strategy against MDR SH on chicken drumsticks at scalding to render safe meat production during subsequent stages of processing.

The overall results from the MS studies indicated that CA could be used as an effective natural antimicrobial against MDR SH in the pre- and post-harvest stages in broiler production and could improve the microbiological safety of chicken meat.

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Literature Review

1.1 Foodborne *Salmonella*

Salmonella is a gram-negative, facultatively anaerobic, non-spore forming motile bacillus belonging to the *Enterobacteriaceae* family. The genus *Salmonella* consists of two species, *S. enterica,* and *S. bongori*. The pathogen causes salmonellosis in humans, characterized by gastrointestinal disease and fever. The CDC estimates 1.2 million infections, 24,000 hospitalizations, and 450 deaths occurring in the United States of America (USA) every year. Immunocompromised persons, children under the age of five, and the elderly are more likely to have severe infections. Symptoms of salmonellosis begin to develop 12 to 72 hours after infection, and those include diarrhea, fever, and abdominal cramps. Severe infections result in lethargy, rashes, and blood in stool and might be fatal (CDC, 2018).

Among the major serovars colonizing poultry such as *S.* Enteritidis and *S.* Typhimurium, *Salmonella enterica enterica* serovar Heidelberg (*S.* Heidelberg) has emerged as a significant *Salmonella* commonly associated with poultry meat or products. Although this serovar has been isolated from poultry products historically, there has been a significant number of foodborne outbreaks occurring recently associated with the pathogen. A significant number of hospitalizations and deaths were reported from *S.* Heidelberg infections linked to the consumption of contaminated ground turkey meat in a 2011 outbreak (CDC, 2011). A multistate outbreak of *S.* Heidelberg linked to broiled chicken liver resulted in 19% hospitalizations (CDC, 2012). In 2014, *S.* Heidelberg caused an outbreak resulting in 22% hospitalizations and a significant recall of 33,840 pounds of mechanically separated chicken at a Tennessee correctional facility (CDC, 2014a). Among

the isolated strains, 22% were resistant to clinically important antibiotics, including beta-lactam inhibitors, cephems, penicillins, tetracyclines, and folate pathway inhibitors (Taylor et al., 2015). *S.* Heidelberg is also reported as the cause of a multistate outbreak with a higher number of systemic infections (15%) linked to the consumption of chicken products sold by a leading poultry company in California, USA. Out of the 68 clinical isolates involved in this outbreak, 35% were multidrug-resistant (MDR) (CDC, 2014b).

Chickens can be infected with *Salmonella* any time during their life and can persist in the gut for a prolonged period. Adhesion of *Salmonella* to the gut epithelium is mediated by several virulence factors, including fimbrial and nonfimbrial adhesins and type III secretion systems, resulting in gut colonization of the pathogen (Rychlik et al., 2014). *Salmonella* can induce inflammatory responses in the cecum of newly hatched chickens. However, as the immune system of the chickens develop later, it will help to resist invasion by the pathogen resulting in the birds continuing as asymptomatic carriers (Rychlik et al., 2014). Although *Salmonella* can be transmitted either horizontally or vertically within the poultry flocks, constant carriage and super shedder status will result in pathogen transmission to humans through contact or by consuming contaminated products (Gopinath et al., 2012).

1.2 Pre-harvest *Salmonella* control strategies in poultry

Some of the commonly employed methods to reduce *Salmonella* in the pre-harvest stages of poultry production include the use of prebiotics, probiotics, plant-derived compounds, and bacteriophages (Patterson and Burkholder, 2003; Atterbury et al., 2007;

Darre et al., 2014). A brief discussion on these control strategies and their mechanisms of action are given below.

1.2.1 Prebiotics

Prebiotics are non-digestible feed ingredients that influence the growth of beneficial bacteria in the host, thereby improve host health. For example, the commonly used prebiotic, mannanoligosaccharide (MOS), can increase the population of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* species and reduce colonization of *Salmonella* in chickens (Fernandez et al., 2010). It can also improve villi height and goblet cells in the jejunum (Baurhoo et al., 2007). Other prebiotics like fructooligosaccharides and isomaltooligosaccharides can increase the amount of short-chain fatty acids like lactate and butyrate in the distal intestine and can reduce *Salmonella* and other pathogens (Micciche et al., 2018).

Some of the major mechanisms of action of prebiotics to eliminate gut pathogens include receptor mimicking, bifidogenic effect, increase concentration of short-chain fatty acids and the number of beneficial bacteria, decrease cecal pH, and immune modulation. Galactooligosaccharide (GOS), produced from lactose with the help of β-galactosidase enzyme, expresses receptor mimicking function, and interacts with the host epithelium to prevent adhesion and invasion by pathogens. It may also have a direct effect on the host by affecting mucus viscosity or inducing a refractory response in the host to prevent invasion (Searle et al., 2009). It has been reported that MOS, an oligosaccharide occurring naturally in the yeast cell wall and different gums (gums arabica), will cause adsorption of bacteria and prevent them from adhering to the intestinal wall (Spring et al., 2000). Some

oligosaccharides like human milk oligosaccharide (HMO) act as a powerful bioactive component of innate immunity and directly affects the production of cytokines and T cell activation (Arslanoglu et al., 2007). Fructooligosaccharide (FOS), produced by either hydrolysis of inulin or enzymatic synthesis from sucrose and xylooligosaccharides (XOS) exerts its antibacterial effect by being bifidogenic (increasing the number of *Bifidobacterium*) (Charalampopoulos and Rastall, 2012). Prebiotics also prime immune response of the host by the production of immunoglobulin A, a major component of mucosal defense against enteropathogens and important in maintaining the integrity of mucosal biofilms and by the robust lymphoid response (Sims et al., 2004).

1.2.2 Probiotics

Probiotics are living microbial organisms that, when administrated in adequate numbers, exert health benefits for the host by improving the host intestinal microbial balance and immunity (FAO-WHO, 2002). Probiotic strains can also be utilized for controlling pathogens, including *Salmonella* in poultry (Tellez et al., 2012). In one of the recent studies, genetically engineered *Escherichia coli* strain Nissle 1917 was reported to reduce *Salmonella* colonization in turkey poults by the production of antimicrobial peptides (Forkus et al., 2017). Different *Lactobacillus* strains were studied to eliminate *Salmonella* from poultry (Thomas et al., 2019). However, a combination of *Lactobacillus* with *Enterococcus* was also found to be effective against *Salmonella* in poultry (Carter et al., 2017). The functional effects of probiotics are mostly by balancing the gut microflora and immune stimulation. Production of organic acids by probiotic bacteria can decrease the gut pH, which is a non-favorable ecological condition for pathogens (Servin et al., 2004).

Probiotics can also produce deconjugated bile acids that exhibit stronger antibacterial activity than bile salts from the host gut. The direct effect of probiotics on bacteria could be due to the production of antimicrobial substances called bacteriocins, which include lantibiotics, microcines, and modified peptides, harboring unusual amino acids like lanthionin (Oelschlaeger, 2010). *Lactobacillus reuteri*, a probiotic bacterium, can produce compounds like reuterin, which is a broad-spectrum antibiotic (Cleusix et al., 2008). Probiotics could modulate the immune system by enhanced production of interleukins and the activity of natural killer cells (Takeda et al., 2006). Cell wall components like teichoic acid from *Lactobacillus* can also involve in anti-inflammatory activity (Grangette et al., 2005). Induction of mucins and defensins by probiotics at mucosal surface causes enhancement of epithelial barrier functions and thereby improve gut health (Shanahan, 2010). Probiotics can also have antitoxic effect by inhibiting the expression of toxins by production of organic acids, destroying toxin by specific antitoxin immunoglobulins (Qamar et al., 2001), restrict the bioavailability of toxins and increased fecal excretion (Turner et al., 2008) or expressing the receptors identical with receptor of toxins (Paton et al., 2006). It was also shown that a probiotic like *Streptococcus thermophilus* could produce a high amount of folate, which is important for DNA repair in epithelial cells (Van Guelpen et al., 2006). The ability of probiotic to bind N-nitroso compounds and heterocyclic aromatic amines is believed to result in the anticancerous effect (Geier et al., 2006).

Adhesion and invasion are considered as important property of gut pathogens to exhibit its pathogenicity. Probiotics can inhibit pathogenic invasion by its secretory factors and adhesion by either induction of mucin production or by competitive exclusion. Also,

probiotics express competitive exclusion either by adhesion to the receptor and blocking the site for pathogen adhesion or by limiting the resources like iron needed for the pathogens (Oelschlaeger, 2010).

When prebiotics and probiotics are mixed, it is called synbiotics, and these combinations can also eliminate harmful bacteria from intestine and improve GI immunity (Sugiharto, 2016).

1.2.3 Organic acids

Organic acids are proven for their beneficial effects. They cause an abundance of *Lactobacillus* and thereby, potentially result in the reduction of *Enterobacteriaceae* members in the lower gut (Saki et al., 2012). Organic acids can be used as water acidifiers during the preslaughter feed withdrawal period. An organic acid mixture (tannic acid, lactic acid, butyric acid, and acetic acid) added in drinking water was found to be effective against the horizontal transmission of *S.* Enteritidis in poultry (Jarquin et al., 2007). However, organic acids failed to decrease *S.* Enteritidis count in crop and cecal tonsils when administered alone, but a significant reduction was observed when supplemented in combination with probiotics (Wolfenden et al., 2007). Reduced water consumption and carcass shrinkage could be the drawbacks while using organic acids in poultry. However, the low concentration of the mixture of organic acids rather than a higher concentration of single acid could reduce this problem (Wolfenden et al., 2007). It is believed that organic acids can cause bacterial death by several mechanisms, which include acidification of cytoplasm, cytoplasmic membrane damage, and energy depletion (Ricke, 2003).

1.2.4 Enzymes

Exogenous enzymes could improve effective preharvest strategies to control *Salmonella* in poultry (Amerah et al., 2012). Exoenzymes in the feed are known for their beneficial effect through increasing digestibility and nutrient availability and improving gut health and litter quality. Carbohydrases, proteases, phytases, and lipases are used for this purpose.

Phytases are known for removing antinutritional factor such as phytates from the gut and improving the digestibility of minerals as well as increasing mucus production (Guilherme et al., 2008). Xylanase supplementation can reduce the horizontal transmission of *S.* Heidelberg in broiler chickens (Amerah et al., 2012). Xylanases increase lactic acid concentration in the ileum and favor better intestinal health and produce propionate in the cecum, which is also toxic to pathogens (Hinton et al., 1993). Supplementation of arabinoxylooligosacharides with endoxylanases protects against *S.* Enteritidis infection in poultry (Eeckhaut et al., 2008). Small fragments of arabinoxylooligosaccharides (Patterson and Burkholder, 2003) and oligosaccharides from the degradation of non-starch polysaccharides (NSPs) have been reported to have a prebiotic effect in the ceca (Persia et al., 2002).

1.2.5 Plant-derived compounds

Plant-derived compounds also have a broad spectrum of activity against enteric pathogens of poultry. *Trans*-cinnamaldehyde, an aromatic aldehyde extracted from the bark of cinnamon, and eugenol, obtained from clove oil were found to be effective against *S.* Enteritidis when therapeutically supplemented through feed for 5 days before slaughter

in broiler chickens without adversely affecting the feed consumption, bodyweight, normal endogenous flora and cecal pH (Kollanoor-Johny et al., 2012a). Carvacrol and thymol, major ingredients in oregano oil, were also found to be effective against *S.* Enteritidis in cecal contents *in vitro* (Kollanoor Johny et al., 2010) and *Campylobacter jejuni* in the cecum of broiler chickens when supplemented through feed (Arsi et al., 2014). Lemongrass essential oil was also found to be effective against *Salmonella in vitro* with citral, the predominant compound of this oil, exhibiting higher activity than the essential oil itself (Shin, 2005). Plant-derived compounds like eugenol, thymol, and carvacrol are also effective in reducing *S.* Enteritidis adhesion and invasion into chicken oviduct and macrophage survival *in vitro* (Upadhyaya et al., 2013).

The mechanisms of action of plant-derived compounds are not completely known in poultry but hypothesized due to their efficacy to inhibit energy metabolism and glucose uptake in bacteria (Gill and Holley, 2004). These compounds also inhibit key enzymes like amino acid decarboxylases (Wendakoon and Sakaguchi,1995). They could also impart changes in the lipid membrane of the bacterial cell, leading to increased membrane permeability and loss of ions (Sikkema et al., 1994; Ultee et al., 2002). Other potential mechanisms involve the downregulation of genes responsible for an outer membrane protein, bacterial motility, and virulence-associated with *Salmonella* pathogenicity island -1 and type III secretion systems (Kollanoor Johny et al., 2017).

1.2.6 Bacteriophages

Bacteriophages are viruses that infect and replicate within bacteria, resulting in bacterial death. Phages share a common ecology with the respective bacterial host and

could be isolated from sources like skin, feces, intestine, and farm environment (Tiwari et al., 2014). Most of the known bacteriophages interact with a specific binding site on target bacteria. This specificity has an advantage over the antibiotics; a dysbiosis would not occur in bacteriophage treatments unlike antibiotic treatments where potential fluctuations in the inherent microbiome populations could occur (Sulakvelidze et al., 2001). At the same time, this uniqueness for a target makes it a challenge for phage therapy due to a narrow spectrum of activity in the host (Joerger, 2003). There are some limitations in using phages as an alternative to antibiotics against enteropathogens. These include phage clearance by host immune systems, development of phage resistance in bacteria, and inactivation of phages by adverse factors of intestinal tract like digestive juices, enzymes, and variations in pH (Gordon Greer, 2005). The basic mechanism of action of bacteriophages is the lytic cycle in which the phages attach and inject phage DNA into the bacteria shutting off the synthesis of host components, and the replicating phage DNA leading to lysis of bacterial cells and release of phages. The other category of phages called lysogenic phages integrates their DNA into host DNA before replication, which may cause the horizontal transfer of genes responsible for bacterial toxin production and antibiotic resistance. Hence selection of phages is important (Sulakvelidze et al., 2001).

Studies have been conducted using bacteriophages against foodborne pathogens at both pre-harvest and post-harvest production in poultry. Bacteriophage Φ 151 from the Myoviridae family and Φ 10 from the Siphoviridae family are found to be effective in reducing *S.* Enteritidis and *S*. Typhimurium by 4.2 and 2.9 $\log_{10}$ CFU/g, respectively after 24 hours of injection of phages with antacids (Atterbury et al., 2007). When lytic phages

CNPSA1, CNPSA3, and CNPSA4, isolated from free-range layers, were used against *S.* Enteritidis PT4 in chickens, a reduction of 3.5 $\log_{10}$ CFU/g was observed (Fiorentin et al., 2005). The reduction of *Salmonella* on chicken skin was found to increase with the increase in the multiplicity of infection when phages, P22 and P29C, which have specificity to common lipopolysaccharide (LPS) antigen, were used (Goode et al., 2003). A phage cocktail of UAB_Phi 20, UAB_Phi 78, and UAB_Phi 87 were effective against *S.* Enteritidis and *S.* Typhimurium on egg and chicken meat (Spricigo et al., 2013).

Bacteriophages may also provide a natural, non-toxic, and feasible method for the preharvest control of *Salmonella*. Continuous administration of bacteriophage may lead to resistance of *Salmonella*, but the higher reduction was obtained when a single high dose of bacteriophages was used in the feed (Fiorentin et al., 2005). The bacteriophage cocktail produced a faster and longer decrease in *Salmonella* concentration than when tested individually (Bardina et al., 2012).

1.3 Post-harvest applications

Once poultry arrive at the slaughterhouse from a farm in transport cages, they are unloaded and hung on shackles. Stunning renders poultry unconscious by either electrical, mechanical, or chemical methods, and bleeding ensures the death of birds. After bleeding, poultry carcasses will be dipped in scalder water. Scalding prepares carcasses for defeathering. After the feathers are removed with the picking device, the carcasses will be ready for evisceration, the process of removal of internal organs. Intestinal and crop rupture in the evisceration stage could result in external and internal contamination by pathogens,

which persist in the subsequent stages even after inside-outside bird washing (Smith et al., 2007). The purpose of the final chilling is to reduce the temperature of the carcass to 4^{o}C within 4 to 8 hours. Sanitizers added at the chilling stage help to reduce pathogen load in the final product.

Even though the flocks are treated with various preharvest strategies during production, feed withdrawal before slaughter and stress from loading can increase the number of *Salmonella* in the crop and cecum (Ramirez et al., 1997). Moreover, transportation to slaughterhouse and scalding are critical stages that cause cross-contamination and could contribute to higher prevalence of *Salmonella* on the carcasses during processing steps (Reiter et al., 2007). Since cross-contamination of pathogens can occur during all stages before chilling of carcasses, chill, and post-chill antimicrobial treatments could also result in improved safety of chicken meat products (USDA FSIS, 2015).

Physical interventions such as the use of UV irradiation, high-pressure treatment, and freezing were tested against *Salmonella* on the chicken meat. Studies have shown that there was little effect of UV irradiation on chicken meat with or without skin (Kim et al., 2002). High-pressure processing could improve the shelf life of chicken breast fillet, but the effect on *Salmonella* depended on inoculation level and storage conditions (Argyri et al., 2018). Freezing is a method of food preservation to lower the temperature and minimize microbial, biochemical, and chemical changes in food. However, crust freezing was found to be least effective against foodborne pathogens like *Salmonella* and *E.coli* (Chaves et al., 2011). Other physical interventions such as the use of electrolyzed oxidizing water and

ozonated water as spray wash and submersion treatments were found to be least effective against *Salmonella* at chilling temperature (Fabrizio et al., 2002).

Chemical interventions are also tested against *Salmonella* on chicken carcasses. Organic acids like acetic acid, citric acid, propionic acid, lactic acid, and malic acid applied during simulated chilling, scalding, and post-chill stages were found to reduce *Salmonella* on chicken skin (Tamblyn and Conner, 1997). A concentration-dependent antibacterial activity of organic acids was observed. Antimicrobial treatments were most effective in the scalders and least effective with post-process dip application (Tamblyn and Conner, 1997). On the other hand, inorganic acids like sodium bisulfate and quaternary ammonium compounds like cetylpyridinium chloride have also been evaluated against *Salmonella* on whole carcasses resulting in a reduction of 2.4 and 1.6 $\log_{10}$ CFU, respectively (Yanbin et al., 1997).

Essential oil application in several poultry processing steps is a new trend in research to ensure food safety. Use of pimenta essential oil reduced *Salmonella* on turkey skin by >2 $\log_{10}$ CFU/sq. in. under scalding and chilling conditions (Nair and Kollanoor Johny, 2017). Oregano essential oil with the modified atmospheric packaging reduced *Enterobacteriaceae* counts and extended the shelf life of chicken breast stored at 4^{o}C (Chouliara et al., 2007)

Biological interventions, including the use of bacteriocins, derived from probiotic bacteria, is yet another potential approach in the post-processing stages. For example, the efficacy of packaging film coated with nisin, a bacteriocin derived from a dairy probiotic, *Lactococcus lactis* was found to reduce *Salmonella* on drumsticks and broiler skin with

improved shelf life (Natrajan and Sheldon, 2000). Bacteriophages were evaluated during the washing process before packaging and found to result in a reduction of 0.9 to 2.2 $\log_{10}$ CFU/g *Salmonella* in chicken meat after storage at 4^{o}C (Spricigo et al., 2013). Similarly, in another study, lytic phages applied at a higher multiplicity of infection caused a maximum reduction of *Salmonella* on chicken skin (Goode et al., 2003).

Although chilling tanks are critical application points for antimicrobials, *Salmonella* that firmly attach to the carcasses results in reduced efficacy of antimicrobial application in the chiller tanks compared to scalding treatments (Tamblyn et al., 1997). Scald additives were found to be effective in reducing the contamination of carcasses (McKee et al., 2008) and could potentially increase the safety of the product. However, due to a high organic load, including feathers, blood, and fecal matter, more investigations are needed to find out a safe and efficacious antimicrobials that act against *Salmonella* present in scalding water. It is reported that common antimicrobials such as chlorine become less effective in water containing organic matter (Bauermeister et al., 2008a).

1.4 Peracetic acid in poultry processing

Peracetic acid (PAA) is a clear colorless liquid soluble in water and has a strong, pungent acetic acid odor with a pH<2. Peracetic acid is an organic oxidizer, and its oxidation potential is higher than chlorine. Compared to the halogenated disinfection by-products from other disinfectants, PAA mainly produces carboxylic acids that are not mutagenic and carcinogenic in waste effluents and surface water used for drinking water (Monarca et al., 2002). For these reasons, PAA is an FDA-approved alternative in various

industries. Besides its use in food processing, it is also used in medical and pharmaceutical industries, and in breweries, dairies, canneries, and wineries as an ideal disinfectant for clean-in-place systems. The compound allows cold sterilization of emulsions, hydrogels, ointments, and powders. It is also used for sterilizing feeds and rooms for gnotobiotic animals. A broad antibacterial spectrum, short exposure time, non-toxic decomposition, and non-objectionable taste and odor from its breakdown products make it a suitable chemical sterilant (Block, 2001).

Peracetic acid is available as a mixture containing acetic acid, hydrogen peroxide, peracetic acid, and water (Gehr et al., 2003). Although there are different methods to produce PAA, including natural photochemical reactions (formaldehyde and oxidative radicals), it is produced industrially by oxidation of acetaldehyde and is approved for used by the USDA under the National Organic Program in livestock production and food handling as a sanitizer, bactericide, and fungicide (USDA AMS, 2016). The reduction of bacteria as a result of PAA addition is generally higher with a rise in temperature and decreases with the biochemical oxygen demand (Stampi et al., 2001). Biochemical oxygen demand (BOD) is the amount of dissolved oxygen needed by aerobic organisms to break down organic material present in a given water sample at a certain temperature over a specific period. Disinfection efficiency of a compound will increase with a decrease in BOD (U.S. EPA, 2003). One of the practical difficulties in the use of PAA is its higher cost, which is four to five times higher than the cost of chlorine (Kitis, 2004).

The pK_a value of PAA is 8.2 at 20^{o}C (USDA AMS, 2016). The pKa value (negative $\log_{10}$ of the acid dissociation constant, Ka) is a method used to indicate the strength of an

acid (Kitis, 2004). The smaller the value of pKa, the stronger the acid. The lower value indicates that the acid dissociates better in water and will have decreased efficiency under alkaline conditions. The efficacy of PAA was found to be higher against fecal coliforms under neutral or mildly acidic conditions (Baldry and French, 1989). The activity of PAA is affected by pH, and the undissociated PAA is considered to be the biocidal form (Kitis, 2004). Under alkaline conditions, the predominance of dissociated form results in low disinfection efficiency. The mechanism of PAA is based on the oxidizing property of hydrogen peroxide. The release of active oxygen causes oxidation of sulfhydryl and sulfur bonds in proteins and enzymes. It is likely that PAA disrupts the chemiosmotic function of lipoprotein cytoplasmic membrane and transport through the rupture of the cell wall (Kitis, 2004).

Peracetic acid has been tested as an antimicrobial for use in poultry chilling tanks. Peracetic acid hydrogen peroxide (PAHP) at 85 ppm (0.0085%) reduced *Salmonella* positive carcasses by 92%, whereas 30 ppm chlorine (0.003%) reduced *Salmonella* by 57%. Additionally, PAHP reduced *Campylobacter* on carcasses exiting the chiller by 43%, while chlorine resulted in only a 13% reduction (Bauermeister et al., 2008a). In another study, 0.02% PAA (200ppm) resulted in a 1.5 and 1 log reduction against *Campylobacter* and *Salmonella*, respectively, and the reduction was more effective than chlorine, which is an industry-standard for chiller applications. In the same study, PAA in chiller did not change flavor appearance and acceptability of chicken meat (Bauermeister et al., 2008b).

Peracetic acid, hydrogen peroxide (HP), and acetic acid (AA) are considered irritant chemicals that can cause acute eye and nasal irritation as well as chronic shortness of

breath. Commonly reported health outcomes due to exposure of PAA while used as a sanitizer/disinfectant include watery eyes, allergy, asthma-like symptoms and dry or sore throat (Hawley et al., 2018). A higher concentration of PAA is a potent skin tumor promoter and weak carcinogen (Bock et al., 1975). However no mutagenic effect has been observed (Yamaguchi and Yamashita, 1980). The compound must be treated with caution to avoid chemical hazards. Contact with organic materials and heavy metal ions of copper, iron, and manganese should be avoided because they can cause decomposition so rapid as to cause ignition and produce fires (Kitis, 2004).

1.5 Caprylic acid

Medium-chain fatty acids (MCFAs) are saturated monocarboxylic acids which comprise of caproic acid (C6), caprylic acid (C8) capric acid (C10) and lauric acid (C12). Supplementation of MCFAs or their triglycerides, especially triglyceryl octanoate has been shown to increase acyl modification of ghrelin, which is important for growth hormone release, appetite stimulation, and metabolic fuel preferences (Nishi et al., 2005). Medium-chain fatty acids also act as an immunostimulant since the compounds can act as ligands for the receptor, which express immune cells and mediate interleukin production (Wang et al., 2006).

Medium-chain fatty acids differ from long-chain fatty acids in several ways. The presence of a comparatively short length of fatty acids make them soluble in an aqueous biological fluid, and the fatty acids can reach the liver directly through the portal vein without the involvement of the lymphatic system. These are also readily available for

oxidation in mitochondria since they can enter the cell without the involvement of transferase enzymes. These characteristics make them useful dietary tools for metabolic diseases like obesity and hypertension (Papamandjaris et al., 1998; Patterson and Burkholder, 2003; De Vogel-van den Bosch et al., 2011). The MCFAs also have cholesterol-reducing effects by enhanced fecal excretion of cholesterol and cholic acids (Xu et al., 2013). They are also known for their immediate availability as an energy source in neonates and young animals. This will help to improve the survival rate and daily weight gain because of their positive effects on intestinal morphology and gut-associated immune system (Zentek et al., 2011).

Evans et al. (2017) evaluated the efficacy of MCFAs for reducing *S.* Typhimurium in the turkey poults. In this study, they supplemented 1.5, 3, 4.5, or 6 pounds of MCFA per ton of feed. Bioluminescence imaging from Meckel's diverticulum to cloaca showed that groups received 6 pounds MCFA/ton of feed resulted in a significant reduction of colonization of *Salmonella* after 3 days of inoculation. Selective enumeration of cecal contents resulted in 1 $\log_{10}$ CFU/g reduction of *Salmonella* compared to a positive control (Evans et al., 2017).

Among the MCFAs, caprylic acid (octanoic acid; CA) has been identified by the industry recently for its many benefits. Caprylic acid is an eight-carbon carboxylic acid, naturally present in bovine milk, breast milk, and coconut oil (Jensen et al., 1990; Marina et al., 2009). It is a Generally Recognized as Safe compound approved by the FDA (CFR 184.1025; AMS, 2016). It is industrially produced by the oxidation of n-octanol or by fermentation and distillation of volatile fatty acids in coconut oil (USDA AMS, 2016). Caprylic acid is known for its broad spectrum of antibacterial activity against both gram-

positive and negative bacteria which are commonly associated with foodborne zoonoses (Nair et al., 2004; Kumar et al., 2005; Solis de Los Santos et al., 2008; Johny et al., 2009). It is recognized as a food contact sanitizer in food handling establishments, and a disinfectant in health care facilities due to its low toxicity, biodegradability, and ubiquitous presence in nature (USDA AMS, 2016). In the presence of hydrogen peroxide, CA forms peroxyoctanoic acid (POOA). Both compounds have a high affinity for fatty tissues than acetic acid and PAA (USDA AMS, 2016). It is also reported that the combination of POOA and PAA has synergistic effects to enhance bactericidal activity when these compounds are used alone (USDA AMS, 2016). One of the treatments used in the current investigation is a combination of CA and PAA to determine if there is any synergistic effect for this combination against *S*. Heidelberg (Manjankattil et al., unpublished data).

1.5.1 Use of CA in preharvest poultry safety

Caprylic acid has proven therapeutic and prophylactic potential against multiple foodborne pathogens. Johny et al. (2009) investigated the prophylactic efficacy of two concentrations (0.7 and 1%) of CA in day-old broiler chicks. The study was conducted for 18 days with a challenge of 5 $\log_{10}$ CFU of *S.* Enteritidis on day 8. The results of this study indicate a concentration-dependent reduction in various parts of the GI tract, liver, and spleen. Johny et al. (2012) also studied the therapeutic efficacy of CA in separate three and six-week trails. In both trials, CA supplemented only for the last five days before sampling. Both concentrations of CA (0.7, 1%) were equally effective in the reduction of *S.* Enteritidis in the liver, spleen, and all part of GI tract except crop, where only 1% CA

brought about a reduction in pathogen compared to the control birds. Both studies resulted in a reduction of approximately 2.5 to 3 $\log_{10}$ CFU/g *S.* Enteritidis in cecum without a significant reduction in bodyweight as well as feed consumption compared to control birds.

Solis de Los Santos et al. (2008) tested CA supplementation in feed against *C. jejuni* in broiler chicks. The authors reported that 0.7% of CA consistently reduced *C. jejuni,* however, higher doses failed to reduce bacterial counts. Solis de Los Santos et al. (2008) also studied the efficacy of CA against *Campylobacter* when supplemented therapeutically in the feed. It was reported that only 0.7 and 1.4% of CA reduced *C. jejuni* by 3 to 5 $\log_{10}$ CFU/g. However, lower and higher concentrations were ineffective in the elimination of bacterial populations. This study also reported an adverse effect on body weight while using 1.4% of CA (de Los Santos et al., 2008).

A twelve hours of feed withdrawal period before the slaughter of broiler chickens is considered as the most critical time when there is a high likelihood for *Salmonella* to grow back due to a lack of suppression of pathogens induced by the antimicrobials. So the administration of CA through water could be an applicable preharvest strategy for improving safety during processing. Metcalf et al. (2011) evaluated the efficacy of sodium octanoate, which is a water-soluble form of CA, against *Campylobacter*. Only 0.175% of sodium octanoate resulted in a 3 log reduction of *Campylobacter* in the cecum. It is believed that a higher absorption rate of water-soluble CA from the intestine might result in the inadequate concentration of CA in cecum to inhibit *Campylobacter* (Metcalf et al., 2011).

In yet another study, Upadhyaya et al. (2015) investigated the benefits of feeding

0.7 and 1% CA in layer chickens after challenging the chickens with *S.* Enteritidis. Supplementation of CA significantly decreased the *S.* Enteritidis populations in shell and yolk without affecting egg production performance, bodyweight, and sensory quality of eggs.

1.5.2 Mechanisms of action of CA

Various mechanisms are reported for the antibacterial property of CA. Sun et al. (1998) proposed the ability of CA to diffuse into the bacterial cell in their undissociated form and dissociate in the protoplasm leading to intracellular acidification in bacteria. Fatty acids can also affect the membrane permeability of the bacterial plasma membrane by incorporating the molecule into the membrane (Bergsson et al., 1998). The specific mechanism of CA against *Salmonella* colonization was studied using invasion assay and determining *hilA* gene expression (Van Immerseel et al. 2004a). Medium-chain fatty acids, including CA, resulted in suppression of *hilA,* a key regulator involved in the *Salmonella* invasion. Kollanoor Johny et al. (2012) found that CA down-regulated *hilA* and *hilD* expression in *S.* Enteritidis. More studies are warranted to determine the mechanism of action of CA in chickens.

1.5.3 Application of CA in foods

Caprylic acid has been tested against major foodborne pathogens in a variety of foods. Monocaprylin, the monoglyceride ester of CA, was effective against *E. coli* O157: H7 in apple juices (Kumar et al., 2005). In another study, a low concentration of CA, along

with citric acid, resulted in a significant reduction of *E. coli* in carrot juice (Kim and Rhee, 2015). Caprylic acid is also a well-known antimicrobial in milk and milk products. A study was conducted to compare the efficacy of different concentrations of CA, and monocaprylin added to milk against *Listeria* and *E. coli*. Both compounds resulted in a significant reduction in pathogens at different temperatures and time points of storage (Nair et al., 2004). The compounds also showed a concentration- and temperature-dependent reduction in *Enterobacter sakazakii* in reconstituted milk formula when heated at three different temperatures after CA treatment (Jang and Rhee, 2009). It is also proven for its effect against *Listeria monocytogenes* in cheese (Gadotti et al., 2014). Caprylic acid, monocaprylin, and sodium caprylate were also found to be effective against fish pathogens, namely *Yersinia ruckeri, Edwardsiella ictaluri, E. tarda,* and *Streptococcus iniae* (Kollanoor et al., 2007) and other shrimp pathogens like *Vibrio* (Immanuel et al., 2011). Riedel et al. (2009) evaluated the effectiveness of CA sodium salt (5%) in chemical decontamination of *C. jejuni* on chicken skin and meat at room temperature. In this study, CA sodium salt resulted in 1.35 $\log_{10}$ reduction of the pathogen on the skin after a minute of dip treatment. They also investigated the effect of an extended decontamination procedure in which the 1-minute dip treatment was followed by 24-hour storage at 5^{o}C, although the magnitude of reduction obtained was similar (Riedel et al., 2009).

Caprylic acid was also tested in combination with other antimicrobials. Moschonas et al. (2012) evaluated the antimicrobial efficacy of CA with carvacrol and polylysine to reduce *Salmonella* contamination in non-RTE, surface-browned, frozen, and breaded chicken products. They found a dose-dependent reduction with all the three individual

antimicrobials, and 1% CA reduced initial pathogen population (4.8 to 4.9 $\log_{10}$ CFU/g) to below the detectable limit. A lower concentration (0.0625%) of CA in combination with either of the other two antimicrobials was found to be effective in reducing 1.8 $\log_{10}$ CFU/g *Salmonella* in the final product after storage. Similarly, a combination of the three antimicrobials at a very low concentration (0.0625%) of CA resulted in 2.4 $\log_{10}$ CFU/g reduced *Salmonella* in the final product after frozen storage (Moschonas et al., 2012). In another study, Hulankova et al. (2013) tested the additive effect of essential oregano oil, citric acid, and CA in vacuum packaged minced beef. A 2.5 $\log_{10}$ reduction of *Listeria* and 1.5 log reduction of lactic acid bacteria were noticed in combination treatments after 10 days of storage at 3^{o}C. However, a negative impact on color and sensory properties was noticed with CA treatment. Studies conducted by Burnett et al. (2006) using CA to reduce *L. monocytogenes* in RTE poultry products observed reduction in the pathogen populations and scoring equivalently in organoleptic evaluation with the controls (Burnett et al., 2007)

1.6 Conclusion and objectives

Foodborne salmonellosis is a major bacterial illness that accounts for a high number of hospitalizations and deaths. Drug-resistant strains of *Salmonella* that cause highly invasive diseases in humans are mainly acquired by humans through the consumption of contaminated poultry meat and meat products. The emergence of MDR strains is considered as an urgent threat to public health and demand to limit the use of antibiotics in the food animal industries, including poultry, has warranted to hasten our search for viable and effective *Salmonella* control strategies. The emerging strains of MDR *S*. Heidelberg is

an example of an imminent threat to human food safety linked to the consumption of contaminated poultry against which immediate control measures are warranted due to the federal curbs of using clinically-important antibiotics.

Preharvest approaches fail to produce *Salmonella*-free carcasses or products at retail because of multiple factors at preharvest and postharvest levels of production. Birds colonized with *Salmonella* in the grow-out houses is the major reason for the high prevalence of the pathogen encountered during processing. In addition, high stress during bird loading and transportation, and feed withdrawal period before slaughter limit the efficacy of alternatives in the gut and favors the multiplication of *Salmonella*. At processing level, the the antibacterial approaches should be able to counteract the already attached *Salmonella* on the carcasses before they are stored and shipped to retail markets.

The use of MCFAs as feed additives is found to be an effective and feasible method among the various alternative approaches to antibiotics for preharvest and postharvest production situations. Caprylic acid, a GRAS-status MCFA, has been tested for its antibacterial efficacy against major foodborne pathogens. The CA intervention applied at preharvest, and post-harvest stages of poultry production could be considered a safe and natural approach to reducing MDR *Salmonella* Heidelberg in broiler chickens and chicken meat. It is previously reported that CA could reduce the colonization of *S*. Enteritidis, a major *Salmonella* serovar, in broiler chicks when supplemented for 18 days and commercial broiler chickens 7 days before slaughter. However, the effect of CA supplemented long-term for 5-weeks through feed against an emerging serovar in chickens, *S*. Heidelberg, is not reported. Moreover, the efficacy of CA for use in processing poultry has not been investigated yet. Therefore, the central hypothesis of this thesis is that CA

would reduce *S.* Heidelberg colonization in the ceca of broiler chickens and on chicken drumsticks during the scalding step. The specific objectives were:

1. To determine the effect of in-feed supplementation of caprylic acid as a preharvest strategy against multidrug-resistant *S.* Heidelberg in the cecum of 5-week-old broiler chickens, and
2. To determine the effect of caprylic acid as processing aid against multidrug-resistant *S.* Heidelberg on chicken drumsticks under scalding conditions

Chapter 2:

To determine the effect of in-feed supplementation of caprylic acid as a preharvest strategy against multidrug-resistant *S.* Heidelberg in the cecum of 5-week-old broiler chickens

Synopsis

Salmonella is the leading bacterial cause of foodborne illness in the United States. *Salmonella* colonizes the chicken cecum and gets excreted through droppings leading to the contamination of the farm environment and poultry carcass during processing. Similar to other major *Salmonella* serovars colonizing poultry, *Salmonella* Heidelberg (SH) is being increasingly isolated from chicken carcasses and recognized as an emerging *Salmonella* associated with foodborne outbreaks through chicken products, recently. In this study, the efficacy of a medium-chain fatty acid, caprylic acid (CA), in reducing SH colonization in the cecum of 5-week old broiler chickens, was investigated. Two independent studies were conducted. In each experiment, day-old chicks were randomly allocated to four different groups (3 chicks/group; two studies). The four groups included in the study were: Negative control (NC), Positive Control (PC), Antibiotic group (AB), and caprylic acid group (CA). The birds in NC and PC were fed with a standard basal diet, whereas the broilers in the AB group received a standard diet containing 50g/ ton bacitracin methylene disalicylate (BMD) for 5 weeks. The CA (1% w/w) was supplemented through the feed to the broilers in the CA group for 5 weeks. All birds except those in the NC group were challenged with 3.69 $\log_{10}$ CFU of multidrug-resistant (MDR) SH (2014 Tennessee correctional facility outbreak) strain by crop gavage. Birds were euthanized 7 days after MDR SH inoculation, by CO_2 asphyxiation. Cecal samples were collected, and the cecal colonization of *Salmonella* was determined after surface plating the homogenates on xylose lysine deoxycholate agar plates. The bacterial counts were transformed to log values, and ANOVA was used for statistical analysis. The BMD supplementation resulted in 3.4 and

4.0 $\log_{10}$ CFU/g reduction of MDR SH in studies 1 and 2, respectively. The CA supplementation also resulted in a comparable reduction in cecal colonization of MDR SH as that of BMD. A reduction of 3.2 and 4.0 $\log_{10}$ CFU/g was observed in studies 1 and 2, respectively, in the CA group compared to PC. Our results indicate that CA could be used as an effective control strategy against MDR SH colonization in 5-week old broilers.

2.1 Introduction

Foodborne salmonellosis is a major public health concern in the USA and worldwide. Approximately 11% of total foodborne illness in the USA are caused by nontyphoidal *Salmonella*, placing them second only to Norovirus, as the major foodborne pathogen affecting humans, resulting in significant number of hospitalizations (35%) and deaths (28%) (Scallan et al., 2011). Poultry serve as a natural reservoir host for *Salmonella*. *Salmonella* can colonize the entire GI tract, with maximum colonization in the cecum as experimentally studied in *S*. Enteritidis-infected broiler chickens (Johny et al., 2009). Fecal shedding of *Salmonella* can contaminate the farm environment, and eggs, and poultry meat during processing.

The resistance of *Salmonella* strains associated with foodborne outbreaks is one of the major concerns, and it is estimated that approximately 100,000 drug-resistant infections are reported every year from drug-resistant *Salmonella*. Antibiotic use in food animals can result in the development of resistance in *Salmonella*. It is reported that about 5% of non-typhoidal *Salmonella* tested by CDC are resistant to five or more classes of antibiotics (CDC, 2013). *Salmonella* enterica serovar Heidelberg (SH) is one among the common nontyphoidal *Salmonella* serotypes that cause an increased risk of illness in humans due to the high invasiveness of the strains (Crump et al., 2011) and drug resistance (Gieraltowski et al., 2016). Chicken products act as an important source of human infections with SH resistant to extended-spectrum cephalosporins (Folster et al., 2012; Gould et al., 2013). Given the federal initiatives to phase out antibiotics from production, alternative

interventions have become a necessity in the pre-harvest stages to reduce *Salmonella* load in the GI tract of birds entering the processing chain.

There are multiple preharvest approaches being tested against *Salmonella* in poultry that includes probiotics, prebiotics, vaccination, and bacteriophages. The use of lactic acid bacteria as probiotics has been reported to reduce *Salmonella* counts in poultry cecum (Pascual et al., 1999; Prado-Rebolledo et al., 2017). Studies have also proven that lactic acid probiotics can reduce the dissemination of *S.* Heidelberg in turkey poults (Thomas et al., 2019). Other approaches, like the use of attenuated strains of *Salmonella* as vaccine strains, and prebiotics like oligosaccharides, also have potential to reduce *Salmonella* colonization in poultry (Azcarate-Peril et al., 2018). Liposome-encapsulated phage administration is another method to reduce *Salmonella* in poultry (Colom et al., 2015). Organic acids, in combination with probiotics, are also reported effective against *Salmonella* in broilers (Wolfenden et al., 2007). Short and medium-chain fatty acids (SCFA, MCFA) are suitable, safe alternatives against *Salmonella.* Among the SCFAs tested, no reduction of *Salmonella* was observed with acetic acid, fumaric acid, and propionic acid. However, butyric acid caused a reduction in colonization of *Salmonella* in cecum without reducing dissemination to liver and spleen (Van Immerseel et al., 2004b).

The MCFAs have potential against *Salmonella* in poultry (Van Immerseel et al., 2004a; Kollanoor Johny et al., 2009, 2012b). Among the four MCFAs (capric, caproic, caprylic, lauric), caprylic acid (CA), an eight carbon MCFA naturally present in breast milk, caprine milk, and coconut oil, is a food-grade chemical approved by the FDA as Generally Recognized as Safe (GRAS) compound. Previous studies reported prophylactic and therapeutic efficacy of CA supplemented through feed to reduce *S.* Enteritidis

populations in broiler chickens (Kollanoor-Johny et al., 2009, 2012b). These studies were conducted either in broiler chicks or in chickens 7 days before slaughter. Given the efficacy of CA against *S.* Enteritidis, this study investigated the effect of 5-week-long supplementation of CA against an MDR outbreak strain of an emerging *Salmonella* serovar, SH, in broiler chickens.

2.2 Materials and Methods

All the methods were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota (Protocol no 1701-34538A; 1710B11901).

2.2.1 Experimental birds and management

Day-old commercial non-vaccinated Ross 708 broiler chicks were procured from a commercial hatchery in Minnesota. Birds were allocated into floor pens at the Poultry Teaching and Research Facility (PTRF) at the University of Minnesota. Age-appropriate temperature, light, and humidity were provided during the entire study period. The birds had access to *ad libitum* feed and water.

2.2.2 Bacterial strain and dosing

SH 1904 (2014 Tennessee correctional facility outbreak strain) was used for inoculating birds. A working culture was prepared from the stock maintained at -80 ^{o}C by transferring it in 10 ml tryptic soy broth (TSB; catalog no. C7141, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) supplemented with 50 µg/mL of nalidixic

acid (NA; CAS no. 3374-05-8, Alfa Aesar, Haverhill, MA, United States) and incubated at 37°C for 24 h. From the second subculture, 1 mL was subcultured in 100 mL TSB supplemented with 50µg/mL of nalidixic acid incubated overnight at 37°C for 24 hours. The culture was sedimented by centrifugation (3600Xg for 15 minutes at 4°C), and the pellet was resuspended in 100 mL PBS and used as inoculum. Bacterial count in the diluted culture was confirmed by plating 100 µL portion of appropriate dilution on Xylose Lysine Deoxycholate (XLD; catalog no. C7322, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) agar with NA and incubating the plates at 37°C for 24 h (Kollanoor Johny et al., 2009).

2.2.3 Experimental design

2.2.3.1 Pre-challenge growth phase

Day-old broiler chicks were weighed and randomly assigned to four groups of 6 birds each. Treatments included a negative (NC) and positive (PC) groups fed a basal diet, the antibiotic group (AB) fed with a basal diet containing BMD at 50 g/ ton of basal diet, CA group fed 1% of the compound through the basal diet. Feed consumption and bodyweight were determined weekly.

2.2.3.2 Challenge phase

At the end of fourth week, all birds were transferred from PTRF to the research animal resources (RAR) facility at the University of Minnesota. Six birds in each group raised in PTRF were divided and placed in two separate pens in the RAR isolator pens. Birds in the groups except NC received 3.69 $\log_{10}$ CFU/5 ml of SH by crop gavage.

Challenge study groups included a negative control (NC; no SH, no CA, no antibiotic), positive control (PC; with SH, no CA, no antibiotic), antibiotic group (AB; with SH, no CA, antibiotic at 50g/ton BMD in feed), and CA group (SH, 1% CA in feed, no antibiotic). After 7 days of challenge, birds from each treatment group were euthanized by carbon dioxide asphyxiation. Necropsy and sample collection were performed at the Veterinary Diagnostic Laboratory at the University of Minnesota.

2.2.4 Determination of *S.* Heidelberg in the cecum

Cecal samples were collected in 10 ml PBS during necropsy and were weighed, homogenized, and serially diluted in PBS. Two hundred microliters of appropriate dilutions were surface plated on XLD + NA plates. Bacterial colonies were enumerated after incubating the plates at 37ºC for 24 h. When the colonies were not detected by direct plating, the samples were tested for surviving cells by enrichment in 10 mL of selenite cystine broth at 37°C for 8 h followed by streaking on XLD + NA plates and incubation at 37ºC for 24 h.

2.2.5 Determination of body weight

Birds were weighed individually at each week. The average bodyweight was determined for each group.

2.2.6 Statistical analysis

A completely randomized design was used to analyze the effect of CA on SH in the cecum. Each pen was considered an experimental unit, and each pen had three birds in it. The design included 4 treatments (NC, PC, AB, and CA) and cecal samples from

three birds on day 7 post-inoculation. Colonies were counted, and appropriate dilution factor and cecal weights were applied to obtain bacterial populations per g of cecum. The numbers of SH colonies were logarithmically transformed before analysis. Mixed procedure of SAS was used for analysis, and mean separation was done by the Tukey test. A P value < 0.05 was considered statistically significant.

2.3 Results

No mortality was observed in any group during the study. As expected, *Salmonella* was not detected from the unchallenged groups (NC), indicating that there were not any inherent *Salmonella* colonization. The bodyweights of birds did not differ between the groups ($P<0.05$). The mean final bodyweight (in kg) of birds in NC, PC, AB, and CA groups were 1.9 ± 0.16, 1.5 ± 0.21, 1.8 ± 0.15 and 1.8 ± 0.16, respectively at the end of 5 weeks.

The effect of CA supplementation through feed on SH populations in the cecum is depicted in Figure 1. Cecal samples from PC had 3.4 to 4.0 $\log_{10}$ CFU SH/g of cecum on day 7 post-inoculation. SH populations in the birds treated with 1% CA were significantly reduced by 3 to 4 $\log_{10}$ CFU/g compared to the birds in PC ($P<0.05$). Similarly, BMD supplementation caused a reduction of SH by 3.4 $\log_{10}$ CFU/g compared to the PC groups.

2.4 Discussion

Once *Salmonella* enters the host through the oral route, it can traverse and colonize the entire length of the GI tract, especially in the cecum, which is the major colonization

site in chickens (Allen-Vercoe et al., 1999). Birds colonized with the pathogen will act as a source of infection to healthy incoming flocks via interaction with the infected litter or farm premises. Consequently, the market-age birds that come out of the grow-out houses will lead to processing of carcasses with questionable food safety standards. Adoption of preharvest strategies that control *Salmonella* in the cecum of birds raised in the grow-out houses will help to minimize bacterial contamination of farms and thereby improving food safety. In this study, we investigated the efficacy of supplementation of CA through feed against the emerging MDR SH strain in the cecum of 5-week-old broiler chickens. We found that CA was highly effective against *S.* Heidelberg in commercial broiler chickens, reducing the pathogen populations by approximately 4 log CFU/g without adversely affecting the bodyweight of chickens. Comparable bodyweights in CA supplemented chickens have been reported previously (Kollanoor Johny et al., 2009; de Los Santos et al., 2008; Khatibjoo et al., 2018).

Caprylic acid has been tested against different foodborne pathogens in both *in vivo* and *in vitro* studies. *In vitro* studies showed that CA was highly effective in killing *S.* Enteritidis in chicken cecal contents rapidly (Vasudevan et al., 2005). Prophylactic supplementation of CA through feed reduced *S.* Enteritidis colonization in cecum by approximately 2.5 log CFU/g compared to control birds (Kollanoor Johny et al., 2009). Similarly, the therapeutic supplementation of CA was also tested successfully against *S.* Enteritidis, and a maximum reduction of 3 log CFU/g was found in the cecum (Kollanoor-Johny et al., 2012b).

Caprylic acid has been tested against other major food pathogens like *Campylobacter*. A consistent reduction was observed in *C. jejuni* with 0.7% CA

supplementation through feed (Solis de Los Santos et al., 2008). Likewise, therapeutic supplementation of CA through the feed for 72 hours before slaughter also caused a reduction of 3 to 4 log CFU/g of *C. jejuni* in the chicken cecum (Solis de Los Santos et al., 2008). However, supplementation of sodium octanoate, a water-soluble form of CA through water, resulted in a less and inconsistent reduction of *Campylobacter* potentially due to increased intestinal absorption of the water-soluble form of the compound and reduced the effective concentration of CA available at cecum (Metcalf et al., 2011).

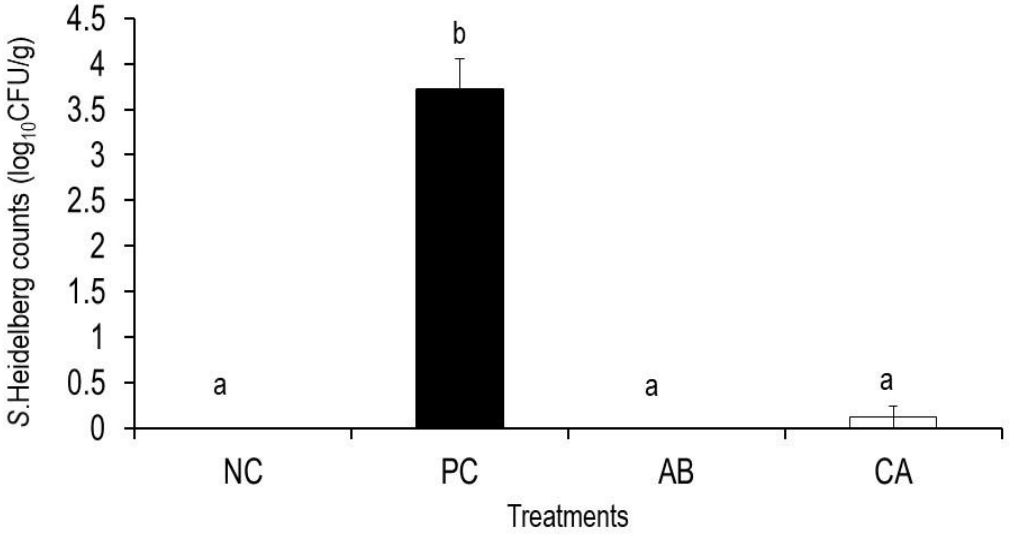
Caprylic acid is considered a safe alternative, with no reported toxicity after oral administration (Traul et al., 2000). Consistent to that, no pathological lesions were detected in liver samples collected from birds supplemented with CA through feed (Kollanoor Johny et al., 2009). Supplementation of CA can also improve meat quality upon storage by delaying lipid peroxidation during storage at refrigeration temperatures (Skřivan et al., 2010). Caprylic acid supplementation also improved feed efficiency, digestibility, and antioxidant properties in piglets (Li et al., 2015). Moreover, CA is also proven for its modulating effect on the microbial ecology and bacterial metabolites in the intestine of pigs (Zentek et al., 2012).

Caprylic acid could exert its antibacterial effect on SH by several mechanisms. Caprylic acid decreased *hilA* expression, which is responsible for the invasion of *Salmonella* to intestinal epithelial cells (Van Immerseel et al., 2004a). Similarly, other studies also found the downregulation of *hilD* and *invF* of *S.* Enteritidis in the presence of CA (Kollanoor-Johny et al., 2012b). The other proposed mechanism CA is its ability to diffuse into the bacterial protoplasm, thereby resulting in intracellular acidification

affecting enzymes and amino acid transport systems adversely (Viegas and Sa-Correia, 1991; Freese et al., 1973; Sun et al., 1998).

In conclusion, this study showed the potential of CA against another major *Salmonella* serotype colonizing chickens, SH. Experimentation of CA supplemented to commercial broiler chickens at field level (translation) has to be the immediate next step validating its efficacy against multiple *Salmonella* serovars colonizing chickens.

Figure 1: Effect of 1% CA supplementation on *S*. Heidelberg populations in the cecum of 5-week old broiler chickens (Mean ± SE, n=6). The error bar indicates standard error. A significant difference was detected when at $P<0.05$



Chapter 3:

To determine the effect of caprylic acid as processing aid against multidrug-resistant *S.* Heidelberg on chicken drumsticks under scalding conditions

Synopsis

Salmonella Heidelberg (SH) is a frequently isolated multidrug-resistant (MDR) *Salmonella* serovar from chicken products. Scalding is a critical control point in poultry processing where the possibility of cross-contamination is high if the incoming carcasses are contaminated with the pathogen. Therefore, effective interventions are necessary at the scalding step to control SH to ensure safe poultry meat processing. The objective of the study was to determine the antimicrobial efficacy of caprylic acid (CA, generally recognized as safe-status medium-chain fatty acid) against MDR SH on chicken drumsticks under soft scalding conditions. Chicken drumsticks were spot inoculated with MDR SH [either lower (~3.0 $\log_{10}$ CFU/g) or higher (5.0 $\log_{10}$ CFU/g) inoculum] and immersed in scalding water containing treatments for 2 min at 54^{o}C (USDA-recommended time-temperature combination for scalding). The antimicrobial treatments included in the study were 0.5% CA, 1% CA, 0.05% peracetic acid (PAA), 0.5% CA + 0.05% PAA and 1.0% CA + 0.05% PAA. Samples inoculated with or without MDR SH and immersed in scalding water containing neither of the antimicrobial treatments served as the positive control (PC) and negative control (NC), respectively. Immediately after scalding, the drumsticks were homogenized in phosphate-buffered saline (PBS), and surviving MDR SH populations were recovered on XLD agar plates (n=6). Similarly, MDR SH populations that survived in scalding water was also determined after surface plating (n=6). Additionally, the efficacy of the scalding treatments against MDR SH survival on drumsticks for a storage period of 48 h at 4^{o}C was determined. Furthermore, the effect of these treatments on the surface color of the drumsticks was also evaluated. The antimicrobial treatments resulted in a significant

reduction of MDR SH on drumsticks. For the lower inoculum, 0.5% CA, 1% CA, 0.05% PAA, 0.5% CA + 0.05% PAA and 1.0% CA + 0.05% PAA resulted in 0.7, 1.0, 2.5, 1.4 and 1.5 $\log_{10}$ CFU/g reduction of MDR SH on drumsticks ($P<0.05$). The same treatments resulted in 0.9, 1.3, 2.5, 2.2, and 2.6 $\log_{10}$ CFU/g reduction of MDR SH when the drumsticks were contaminated with a higher inoculum level ($P<0.05$). Moreover, the antimicrobial treatments completely inactivated MDR SH in scalding water, whereas 2.0 to 4.0 $\log_{10}$ CFU/mL MDR SH survived in PC ($P<0.05$). Also, the scalding treatments were effective in inhibiting MDR SH on the drumsticks compared to the respective controls during a storage period of 48 h at 4^{o}C, although the magnitude of reduction remained the same as observed during the scalding treatment ($P<0.05$). Additionally, none of the treatments affected the color of the drumsticks ($P>0.05$). Results indicate that CA could be used as effective processing aid against MDR SH on chicken products.

3.1 Introduction

Nontyphoidal *Salmonella* is the leading cause of foodborne illnesses, hospitalizations, and deaths caused by harmful pathogens (Scallan et al., 2011). Foodborne salmonellosis results in an estimated 1.2 million infections, 23,000 hospitalizations, and 450 deaths in the US every year. The direct medical cost is estimated to be $3.6 billion annually (USDA ERS, 2014). *Salmonella* outbreaks are commonly associated with poultry-related food sources. Among all recovered *Salmonella* from retail meat, 9.1% was from chicken, and multidrug-resistant (MDR) strains were high in poultry meat ranging from 20 to 36% (CDC, 2014c).

Salmonella Heidelberg (SH) is considered a highly invasive serotype and one among the common ones isolated from animal products. Resistance to critically important antibiotics that are being used as a first-line treatment for human salmonellosis has been reported markedly higher in serotypes associated with poultry (Hoffmann et al., 2012). Recent studies showed that SH isolated from imported poultry meat had MDR profiles, including extended-spectrum cephalosporins and fluoroquinolones (Campos et al., 2018).

To reduce the incidences of foodborne outbreaks through poultry, interventions should be employed in processing steps as well. In the processing facility, scalding is the first step where stunned and bled birds are exposed to common water bath, hence could play a major role in cross-contamination. Scalding is the process in which carcasses are dipped in hot water to ease de-feathering (removal of feathers). Temperature and time combination is important to prepare carcasses for feather removal. The maximum

temperature and time in soft scald conditions in broiler are 54^{o}C and 120 seconds. Studies have shown that reduction of *Salmonella* at 55^{o}C and 60^{o}C were similar (Yang et al., 2001), but at a higher temperature, carcasses become oily and make easier for *Salmonella* to stick to the surface of the skin. Optimum pH for *Salmonella* growth is 6.5 to 7.5, and making an acidic pH in the scalding water effective in reducing *Salmonella* (Okrend et al., 1986). The presence of high organic matter in scalding water makes the antimicrobials less effective, and studies have been conducted with several organic acids as antimicrobials in the scalding tank to present an acidic environment aiding to control *Salmonella*.

Peracetic acid (PAA) is an organic oxidizer and a mixture containing active ingredients such as hydrogen peroxide and acetic acid. It is found that PAA is affected by organic materials to a lesser degree than chlorine (Bauermeister et al., 2008b). It was tested alone or in combination with other antimicrobials in post-scalding steps (Ri´o et al., 2007; Chen et al., 2014). However, studies on the application of PAA in scalding water have not been done yet due to the high cost involved. Also, there are recent concerns over the use of PAA in processing facilities due to its capacity to cause pulmonary issues in processing facility personnel (Hawley et al., 2018).

Caprylic acid (CA) is an eight carbon, medium-chain fatty acid (MCFA) naturally present in coconut oil and bovine milk (Jensen et al., 1990; Marina et al., 2009). It is recognized as a GRAS status food additive by the FDA. Caprylic acid is proven for its antimicrobial activity against both Gram-positive and Gram-negative bacterial pathogens in different kinds of foods. It has been tested alone or in combination with other essential oils and antimicrobials in meat and meat products (Hulankova et al., 2013; Moschonas et

al., 2012), however, its potential as a processing aid, especially in scalding tanks, has not been explored. Also, the combination of CA and PAA that produces active peroxyoctanoic acid, has not been explored in scalder simulations. Hence, the objectives of this study were to determine the potential of CA alone or in combination with PAA as a processing aid in the scalding step of processing against SH on chicken meat and drumsticks.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

Two strains of MDR SH from a Tennessee correctional facility (1904 and 466) were used in this study. Each strain was cultured separately from the glycerol stock culture stored at -80^{o}C. Working cultures of SH 1904 was prepared by adding 100 µL of stock culture to 10 ml tryptic soy broth (TSB; catalog no. C7141, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) containing nalidixic acid sodium salt (NA; CAS no. 3374-05-8, Alfa Aesar, Haverhill, MA, United States) at 50 µg/mL and incubated at 37^{o}C for 24 hours. The SH 466 strain was pre-induced for resistance to 50 µg/mL of NA to facilitate selective enumeration of the pathogen. Growth of bacteria was determined by the presence of black colonies after plating an appropriate dilution of overnight culture on xylose lysine desoxycholate agar (XLD; catalog no. C7322, Criterion, Hardy Diagnostics, Santa Maria, CA, United States) plates containing 50 µg/mL NA and incubating at 37^{o}C for 24 hours. Bacterial inoculum was prepared from overnight broth culture (approximately 9 $\log_{10}$ CFU/mL) after centrifugation (3600xg, 15 minutes, 4^{o}C) and suspending the pellet in sterile PBS (Kollanoor-Johny et al., 2012). An inoculum level of 2 to 3 $\log_{10}$ CFU/g SH

1904 was used for the study on meat and drumsticks. A mixture of resuspended pellets from cultures of both strains was used for repeating the study with a higher inoculum of 5 $\log_{10}$ CFU/g and chilling and storage experiments.

3.2.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The bacterial time-kill assay, as well as MIC and MBC determination of CA against SH, were conducted (Kollanoor et al., 2007). Six different concentrations (0.03%, 0.06%, 0.12%, 0.25%, 0.5% and 1%) of CA were prepared in 10 ml TSB containing 50 µg/mL NA. A bacterial inoculum of 5 $\log_{10}$ CFU/mL was prepared from the diluted and resuspended bacterial pellet obtained after centrifugation of the overnight culture of SH in TSB and was used for the study. A 100 µL portion of bacteria was added to each treatment and incubated at 37^{o}C for 24 hours. A control sample containing inoculated TSB with no CA was also included. Surviving populations of bacteria were enumerated at 0, 8, and 24 hours of incubation after plating appropriate dilutions of bacteria on XLD + NA agar. Samples were also tested for surviving bacteria by enriching 1 mL of sample in 10 ml selenite cystine broth (SCB; Criterion, Hardy Diagnostics, Santa Maria, CA, United States) at 37^{o}C for 24 hours and streaked on XLD plates with NA. Black colonies on XLD plates after 24 hours of incubation was considered as enrichment positive. The study was repeated six times.

3.2.3 Preparation of treatments at simulated scalding conditions

Caprylic acid (CA; Sigma Aldrich, St. Louis, MO, USA) treatments (0.5, 1, and

2%) were prepared by mixing the appropriate amount of the compound in autoclaved tap water and vortexing for 30 seconds. Two concentrations (0.05% and 0.12%) of PAA (Perasan MP 2; Envirotech, CA, USA) and different combinations of the two compounds (0.05% CA + 0.05% PAA, 0.1% CA + 0.05% PAA, 2% CA + 0.12% PAA, 1% CA + 0.12% PAA) were also prepared in autoclaved tap water and vortexed thoroughly (100/125V, 50/60Hz; Neutec Group Inc., 200 central ave, NY, United States) for 30 seconds. The temperature of treatment water was set at 54^{o}C in a water bath to simulate the scalding steps in poultry processing.

3.2.4 Determination of the effect of CA and PAA against MDR SH on chicken breast fillets

A study was conducted with retail natural chicken breast fillets (boneless, skinless, 99% fat-free, NAE commercial brand) purchased from a local store to determine the effect of adding CA and PAA in scalding water against SH. Meat samples were cut into 25 g pieces and exposed to UV light for 15 minutes in a biosafety cabinet to remove surface bacteria before inoculation (Nair and Kollanoor Johny, 2017). Different concentrations of CA (1 and 2%), PAA (0.12%) and their combinations (1% CA + 0.12% PAA and 2% CA + 0.12% PAA) were prepared in 250 ml autoclaved tap water maintained at 54^{o}C and vortexed for 30 seconds. PAA at 0.12% was chosen in this study because 0.12% is the recommended maximum dose for meat. Meat samples were spot inoculated with a diluted culture of SH at a dose of 2.8 to 3 $\log_{10}$ CFU/g and spread all over the meat samples with a glass rod. After providing 20 minutes of attachment time, each meat sample was separately dipped in treatments maintained at 54^{o}C for 2 minutes. Meat samples were

homogenized in 250 ml PBS for 1 minute immediately after the dip treatment. The survival rate of the pathogen was determined in both treatment water and meat samples. Autoclaved tap water without any treatments maintained at 54^{o}C and 20^{o}C inoculated with the pathogen were kept as positive control (PC). An uninoculated meat sample dipped in autoclaved tap water was used as a negative control (NC).

3.2.5 Determining the antibacterial effect of CA and PAA against MDR SH on chicken drumsticks under simulated scalding conditions

All-natural, fresh retail chicken drumsticks (commercial brand) were obtained from a retail store, weighed and exposed to UV light in a biosafety cabinet for 15 minutes to kill the microflora before inoculating with SH. A lower (2 to 3 $\log_{10}$ CFU/g) and a higher inoculum (5 $\log_{10}$ CFU/g) levels were tested as two sets of the experiments. Treatments were prepared in 350 ml sterile tap water at 54^{o}C and vortexed for 30 seconds for uniform mixing. After providing 20 minutes of pathogen attachment time, the drumsticks were dipped in the treatment solution for 2 minutes. Samples were transferred to 350 ml PBS in whirl pack bags and mixed well by rigorous shaking of bags with hands for 60 seconds. Uninoculated drumstick samples dipped in tap water served as NC, and inoculated samples dipped in sterile tap water without any treatments and maintained at 54^{o}C and 20^{o}C were serving as PC.

3.2.6 Effect of CA and PAA applied in scalding water against MDR SH survival after chilling and 2 days storage

The selected effective concentrations (1% CA, 0.05% PAA, and 1% CA + 0.05%

PAA) were used to prepare treatment water for the experiments. PAA at 0.05% was selected after preliminary study with different concentrations in scalding conditions (data not shown). A cocktail of two strains of SH at a final concentration of 5 $\log_{10}$ CFU/g was inoculated on the drumsticks. The experiment was repeated as in the previous study until dipping. After 2 minutes of dipping meat samples in the treatment solutions, they were transferred to 350 mL autoclaved tap water maintained at 4^{o}C for 30 minutes to simulate chilling conditions. After 30 minutes of chilling, samples were transferred to 350 mL PBS and mixed well for 60 seconds, and SH survival was determined (Nair et al., 2018) in both chilling water and on drumstick samples. After chilling treatment, an additional set of sample was transferred to whirl pack bags and were kept at 4^{o}C for 48 hours. The surviving SH populations were determined after two days of incubation by surface plating and enrichment.

3.2.7 Microbiological Analysis

Survival of SH populations on chicken drumsticks and dipping water was determined by the broth dilution method. After homogenizing the samples, 1 mL of rinsates was serially diluted 10-fold, and 200 µL from appropriate dilutions and was surface plated on XLD + NA plates. Black-colored SH colonies were enumerated after 24 h of incubation at 37^{o}C. One ml each of the dipping solution and meat rinsate was enriched in 10 mL SCB, incubated for 8 hours, and streaked on XLD + NA plates for detection of bacteria when not detected by direct plating.

3.2.8 Determination of treatment water pH

The pH of treated water used for the study with higher inoculum was measured by placing the probe of pH meter (Symphony B10P, VWR, Radnor, PA) into the sample in PBS after microbiological analysis.

3.2.9 Effect of CA and PAA on the surface color of chicken drumsticks

Color changes on the drumsticks after 2 minutes dipping in treatments at scalding temperature were measured using hunter handheld colorimeter (Hunter Lab MiniScan EZ 4500S Spectrophotometer, Reston, VA). The L* (lightness), a* (redness), and b* (yellowness) values were recorded from six different points of a single drumstick after calibrating the chroma meter (Nair et al., 2018).

3.2.10 Statistical analysis

A completely randomized design was used to determine the effect of CA and PAA against SH in all experiments. Experiments were repeated six times, and each sample (either meat or drumsticks or treatment water) served as an experimental unit. The number of bacterial colonies was logarithmically transferred before analysis to achieve homogeneity of variance. The samples from which no bacteria were detected after direct plating but positive after enrichment, were assumed value of 0.90 for analysis, the data were analyzed using the PROC-MIXED procedure of SAS. Differences among the means were detected using the Tukey test. A P value of < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Effect of CA against MDR SH in broth culture

As expected, MDR SH in the control group grew from 6 to 9 $\log_{10}$ CFU/ml over 24 hours of incubation at 37^{o}C (Figures 1A-C). Concentrations of 0.03%, 0.06%, and 0.12% CA resulted in a marginal but significant reduction in bacterial growth compared to the PC over time (Figures 1A-C; $P<0.05$). Whereas 0.25% resulted in 4 log reduction of SH compared to PC after 8 hours of incubation (Figure 1B), all concentrations above it resulted in a rapid reduction of MDR SH to undetectable levels even after 30 seconds of treatment (Figure 1A-C). The reduction of MDR SH continued for 24 hours after incubation. The MIC and MBC of CA against MDR SH were 0.25% and 0.5%, respectively (Figure 1A-1C).

3.3.2 Effect of CA, PAA and their combinations against MDR SH on chicken breast fillets in scalding water

All treatments resulted in a significant reduction in MDR SH on breast fillets compared to the PC (Figure 2A). The bacteria in PC at both temperatures grew to 2.5 to 2.7 $\log_{10}$ CFU/ml (Figure 2A). There was no significant difference between MDR SH populations on the breast fillets dipped in water maintained at 54^{o}C and 20^{o}C among the controls. All treatments significantly reduced MDR SH populations compared to PC. Among the treatments, 0.12% PAA and a combination of 2% CA and 0.12% PAA caused similar numerical reduction, which was 1.25 $\log_{10}$ CFU/g lower compared to PC. The combination of 1% CA and 0.12% PAA resulted in maximum reduction (1.57 $\log_{10}$ CFU/g;

P<0.05) compared to PC, although these treatments were not significantly different from 0.12% PAA or the combination of 2% CA and 0.12% PAA groups (P>0.05). All treatments eliminated MDR SH populations in the scalding water to undetectable levels, while PC group had 1.4 $\log_{10}$ CFU/mL of SH (Figure 2B).

3.3.3 Effect of CA, PAA and their combinations against MDR SH on chicken drumsticks in scalding water

A lower (3 $\log_{10}$ CFU) and a higher inoculum (5 $\log_{10}$ CFU) of MDR SH were used in these studies (Figures 3 and 4, respectively). There was no effect of temperature on MDR SH survival on drumsticks. Both PCs had 2.5 $\log_{10}$ CFU of bacteria attached on them. With the lower inoculum, 0.05% PAA resulted in 2.5 $\log_{10}$ CFU reduction of MDR SH followed by the combination of CA and PAA treatments. The addition of CA at 0.5% and 1% caused a reduction of 0.69 and 0.98 $\log_{10}$ CFU, respectively, compared to PC (Figure 3A), and the reduction of MDR SH was slightly higher (1.36 and 1.52 $\log_{10}$ CFU) when PAA was combined with CA at these concentrations (Figure 3A).

When a higher inoculum was used, 0.05% PAA could still result in ~2.5 $\log_{10}$ CFU reduction of MDR SH, compared to PCs (Figure 4A). The addition of CA resulted in reduction of ~0.93 and 1.33 $\log_{10}$ CFU SH respectively, with 0.5 and 1% CA. Similar to the lower inoculum study, no additive effect was observed in bacterial reduction when combinations were used. The combination of 0.5% CA and 0.05% PAA, and 1% CA and 0.05% PAA resulted in 2.21 and 2.6 $\log_{10}$ CFU reduction of MDR SH, compared to PC (Figure 4A).

The PC had 2 and 4 $\log_{10}$ CFU/mL SH in scalding water after dip treatment in the lower and higher inoculum studies, respectively. However, in both studies, no SH was recovered from the scalding water even after enrichment (Figures 3B and 4B).

3.3.4 Effect of CA, PAA and their combinations on MDR SH survival on drumsticks after chilling and storage

The PC had approximately 4 $\log_{10}$ CFU MDR SH on the drumsticks. The addition of 1% CA resulted in ~1 $\log_{10}$ reduction of SH on chicken drumstick after 30 minutes of chilling, which was similar to the reduction obtained immediately after scalding when 3 $\log_{10}$ CFU was applied on the drumsticks (low inoculum study) at the beginning (Figure 5A). This reduction was maintained even after 48 h of storage (Figure 5B). On the other hand, PAA applied at 0.05% resulted in a reduction of 1.25 $\log_{10}$ CFU/g of MDR SH, which was significantly different from the 1% CA group ($P<0.05$; figure 5A). However, this reduction increased to 1.6 log after 48 hours of storage (Figure 5B). The combination of PAA at 0.05% and 1% CA resulted in a significant reduction of ~2 $\log_{10}$ CFU/g after 48 hours of storage (Figure 5B).

All three treatments significantly reduced SH in chilling water compared to PC ($P<0.05$). Reductions of 1.5, 2.4 and 2.25 $\log_{10}$ CFU/ml in chilling water were obtained with treatments of 1% CA, 0.05% PAA, and 1% CA + 0.05% PAA respectively (Figure 5C).

3.3.5 Effect of CA, PAA, and the combinations on the surface meat color of drumsticks after dip treatment at 54^{o}C for 2 minutes

There were no significant differences in L*, a*and b* values of drumstick samples dipped in treatment water maintained at 54^{o}C for 2 minutes (P>0.05; Table 1).

3.4 Discussion

Scalding is the first critical control point in poultry processing since the step could result in the initial attachment of *Salmonella* to the skin and carcasses resulting in cross-contamination of fresh incoming carcasses. Once *Salmonella* gets enough contact time, it will get attached to the carcasses fairly well, resulting in the carryover of contamination throughout the subsequent stages of processing (Mcbride et al., 1980). Hard scalding (59 - 64^{o}C and 30 – 75 seconds) and soft scalding (51 – 54^{o}C and 90 – 120 seconds) are two types of scalding methods commonly employed in broiler processing. A temperature above 47^{o}C is sufficient to control *Salmonella* growth since the organisms cannot grow at and above that temperature (USDA-FSIS, 2015). Yang et al. (2001) found that *Salmonella* counts could be reduced with higher water temperatures (55 – 60^{o}C) and a long duration of dip. In this study, we found that temperature alone cannot significantly reduce *S.* Heidelberg on the skin and in scalding water. A possible reason is that the skin temperature cannot reach up to the temperature of scalding water within the recommended short dip time (Yang et al., 2001). The other approach to reduce *Salmonella* in scalding water is maintaining a pH either below or above the optimum pH for *Salmonella* (6.5 - 7.5). The pH values above 8.5 and below 4 have a profound effect on *Salmonella* (Humphrey et al.,

1981). The mechanism by which these environments are thought to destroy *Salmonella* is by altering functional enzymes (Sun et al., 1998). Hence the combined effect of temperature and pH can be achieved by adding antimicrobials at these stages.

Scald additives containing sodium hydroxide in scalding tanks have reported reducing *Salmonella* Typhimurium on carcasses compared to soft scald and hard scald dip without antimicrobials (McKee et al., 2008). Chlorine is a common antimicrobial in the chilling stage; however, it is not approved in scalders since it will immediately be deactivated by the organic load in the scalder and can gas-off due to a higher scalder temperature (USDA FSIS, 2015). Organic acids were also tested against *S.* Kedougou at 50^{o}C and found that a combination of formic and propionic acids was more effective, followed by lactic acid and acetic acid (Cherrington et al., 1992). In another study that compared the antibacterial effect of different disinfectants in both scalder and chiller, sodium metabisulfite showed no activity in either situation, and chlorine was least effective in scalding. However, both trisodium phosphate (8% or 80000ppm) and acetic acid (5% or 50000ppm) caused a similar reduction of approximately 1.8 to 2 $\log_{10}$ CFU/skin; however, acetic acid at 5% caused discoloration on the skin (Tamblyn et al., 1997).

Lactic acid at 0.25% and potassium sorbate at 2.5% were also found to be effective under scalding conditions against *S.* Typhimurium and *S.* Sofia*;* however, lactic acid treatment under high temperature resulted in undesirable color and texture of the chicken carcasses (Morrison and Fleet, 1985). Succinic acid along with hot water was found to be effective to reduce spoilage organisms (Cox et al., 1974) and *S.* Montevideo (Juven et al., 1974) but heat treatment resulted in changes in odor and gross appearance (Juven et al.,

1974; Cox et al., 1974). As in our study, carcasses that are chilled or stored have already been subjected to the antibacterial treatment of 6% phosphate during scalding caused a reduction of the buildup of *S*. Typhimurium in the chill water and storage (Thomson et al., 1978).

Bauermeister et al. (2008) tested PAA as an intervention strategy in poultry chiller to decrease *Salmonella* and *Campylobacter*. The concentration of 0.02% (200ppm) PAA decreased *S.* Typhimurium by (>1.5 logs CFU/ sample) compared to a chlorine treatment and extended shelf-life of products without compromising organoleptic properties. No studies have been conducted to test the efficacy of PAA under scalding conditions. Mechanisms of action of PAA could be due to acidification and oxidation effects. Strong oxidizing property of PAA disrupts the permeability of cell membrane and alter protein synthesis through reaction with sulfhydryl, sulfides, and nucleotides. Indirect antimicrobial action could occur through acidification of the carcass surface and penetration of undissociated acids into bacterial cells (Oyarzabal, 2005). Caprylic acid also causes intracellular acidification after entering through the lipophilic cell membrane and resulting in the dissociation of the compound into H+ and caprylate ions. The decrease in the pH would result in the reduced or diminished activity of enzymes resulting in bacterial death (Sun et al., 1998).

In this study, the effect of CA against SH on chicken drumsticks under scalding conditions was investigated as a natural processing aid. Both CA and PAA resulted in a significant reduction in SH survival on drumsticks after scalding, chilling, and 48 hours of storage without adversely affecting the surface color of chicken drumsticks. A comparable

reduction of the pathogen with CA was observed. Peracetic acid, although it is a good option, has limitations for use in processing facilities, including its pulmonary irritant nature, neutralization in the presence of organic matter, and evaporation at scalding temperatures (Kitis, 2004; Hawley et al., 2018). Moreover, no SH was detected in the scalding water with PAA and CA groups, while *Salmonella* was present in the control groups. The results indicate that CA could be a natural alternative against SH on chicken carcasses in scalding water; however, validation with the whole carcass, varying age of scalding water, and level of organic content in the scalding water must be carried out before its recommendation for processing use.

Figure 1(A-C): Effect of caprylic acid against *Salmonella* Heidelberg in broth culture at (A) 0, (B) 8 and (C) 24 h of incubation (Mean±SE; n=6). a,b,c – bars with different superscripts are significantly different from each other at P<0.05. PC – Positive control, CA – Caprylic acid

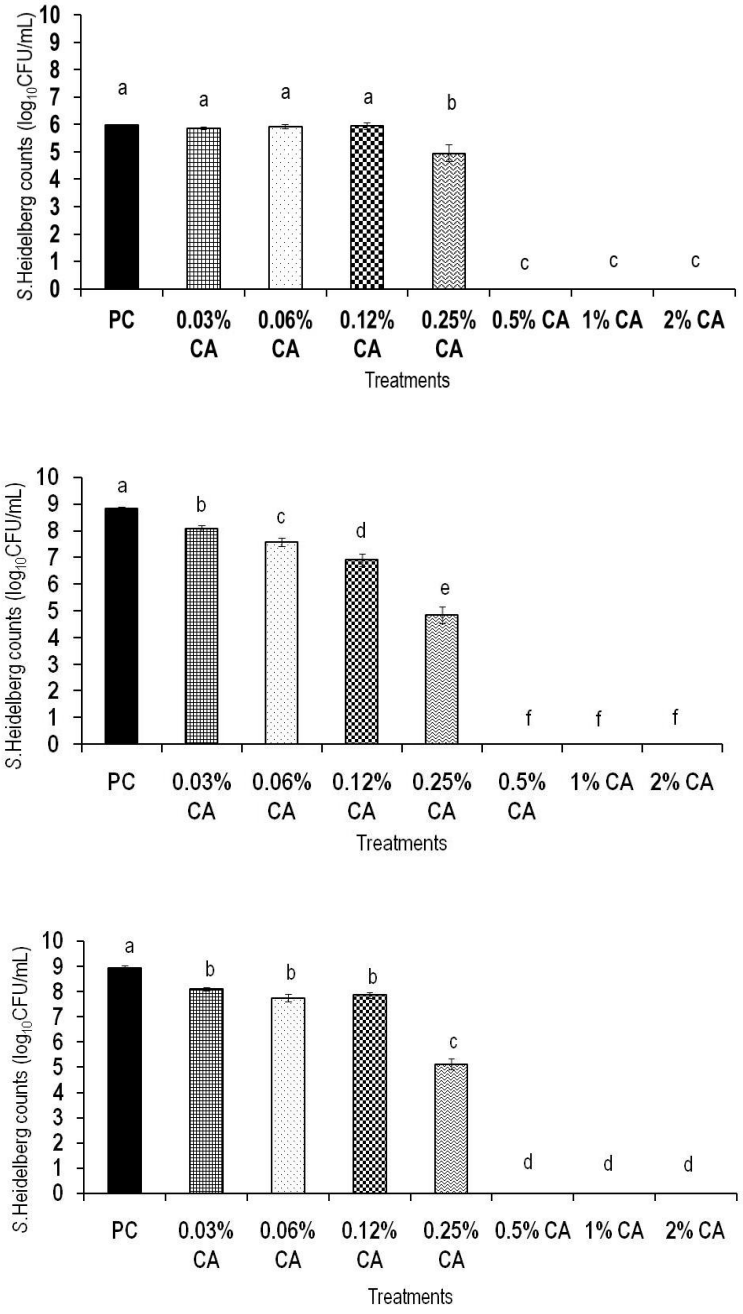


Figure 2A: Effect of caprylic acid against *Salmonella* Heidelberg on chicken breast fillet under simulated scalding conditions (54^{o}C and 2 minutes dip) (Mean ± SE; n=6). a,b – bars with different superscripts are significantly different from each other at P<0.05. PC (20C) – Positive control at 20^{o}C, PC (54C) – Positive control at 54^{o}C, CA – Caprylic acid, PAA – Peracetic acid

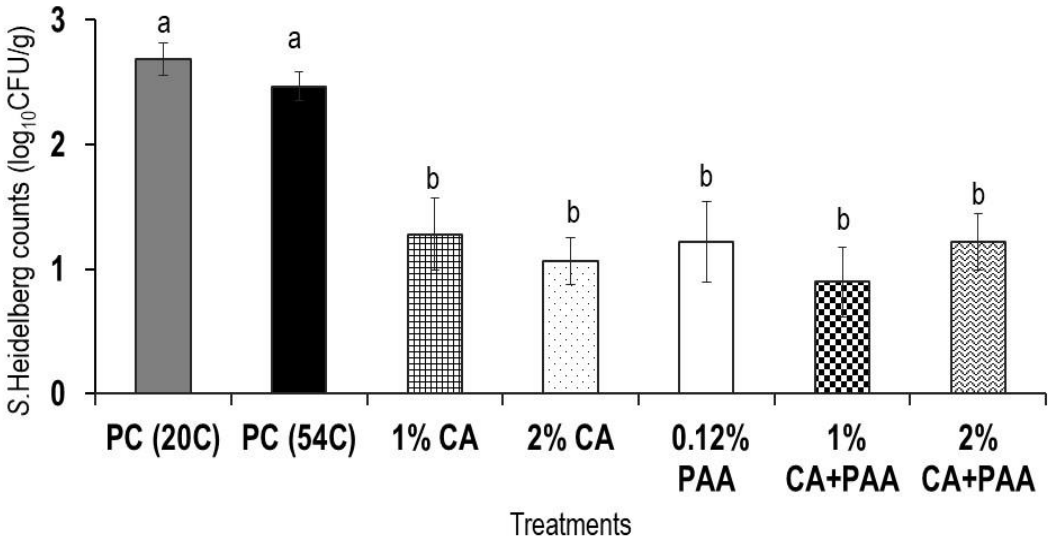


Figure 2B: Effect of caprylic acid against *Salmonella* Heidelberg in scalding water. (Mean ± SE; n=6). a,b – bars with different superscripts are significantly different from each other at P<0.05. PC (20C) – Positive control at 20°C, PC (54C) – Positive control at 54°C, CA – Caprylic acid, PAA – Peracetic acid

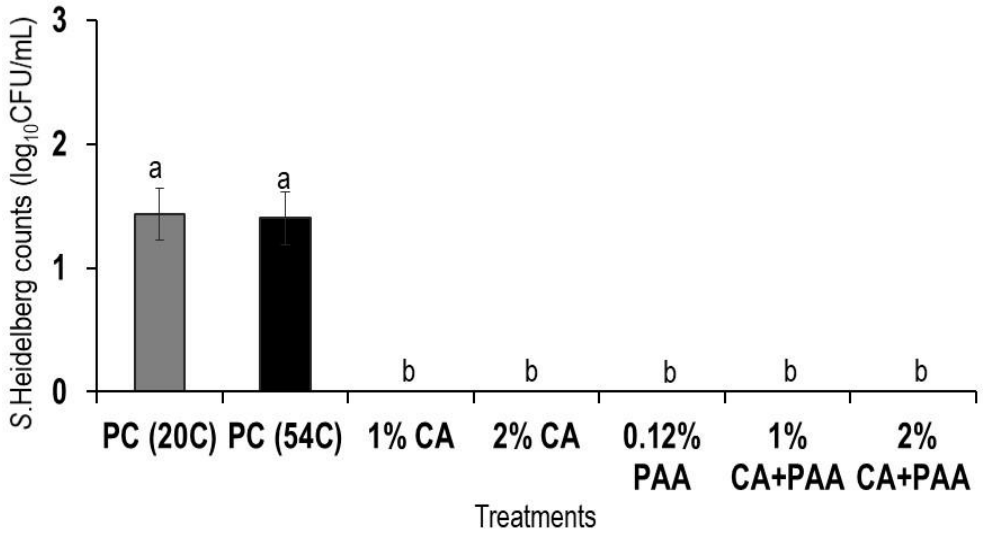


Figure 3A: Effect of caprylic acid against *Salmonella* Heidelberg (low inoculum) on chicken drumsticks under simulated scalding conditions (54^{o}C and 2 minutes dip) (Mean ± SE; n=6). a,b,c, d – bars with different superscripts are significantly different from each other at P<0.05. PC (20C) – Positive control at 20^{o}C, PC (54C) – Positive control at 54^{o}C, CA – Caprylic acid, PAA – Peracetic acid

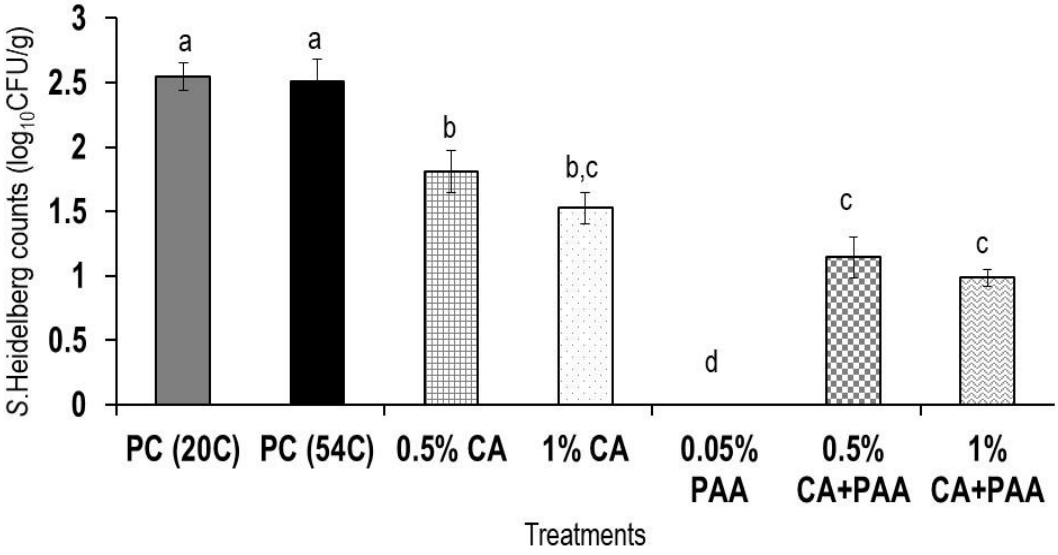


Figure 3B: Effect of caprylic acid against *Salmonella* Heidelberg (low inoculum) in scalding water (Mean ± SE; n=6). a,b – bars with different superscripts are significantly different from each other at P<0.05. PC (20C) – Positive control at 20°C, PC (54C) – Positive control at 54°C, CA – Caprylic acid, PAA – Peracetic acid

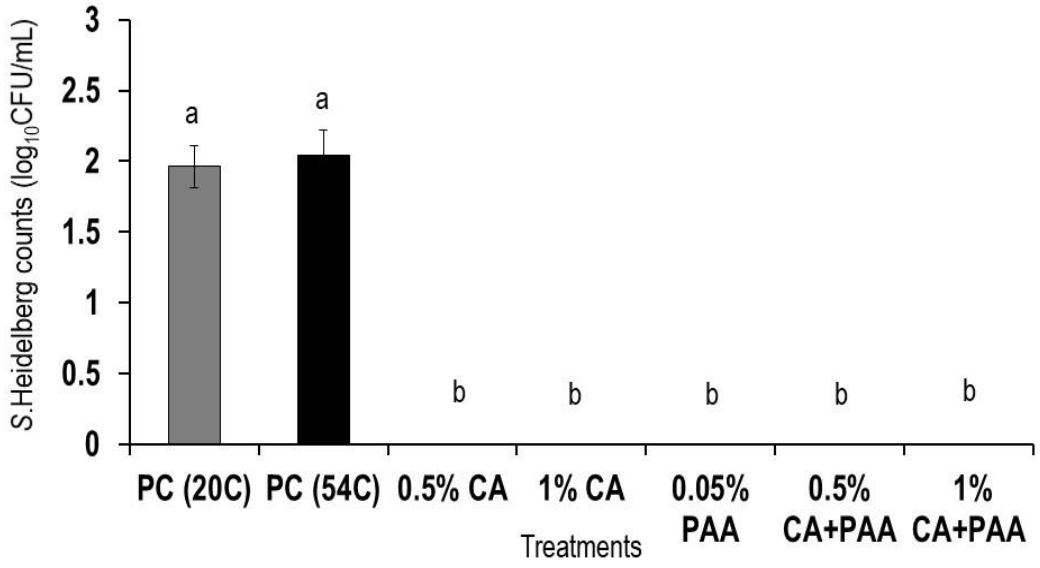


Figure 4A: Effect of caprylic acid against *Salmonella* Heidelberg (high inoculum) on chicken drumsticks under simulated scalding conditions (54^{o}C and 2 min dip) (Mean ± SE; n=6). a,b,c – bars with different superscripts are significantly different from each other at P<0.05. PC (20C) – Positive control at 20^{o}C, PC (54C) – Positive control at 54^{o}C, CA – Caprylic acid, PAA – Peracetic acid

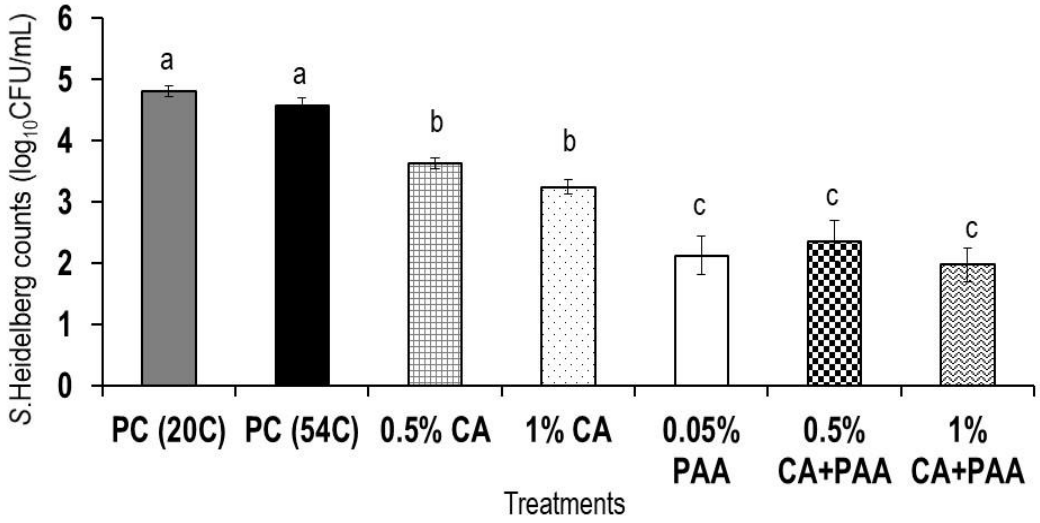


Figure 4B: Effect of caprylic acid against *Salmonella* Heidelberg (high inoculum) in scalding water (Mean ± SE; n=6). a,b – bars with different superscripts are significantly different from each other at P<0.05. PC (20C) – Positive control at 20°C, PC (54C) – Positive control at 54°C, CA – Caprylic acid, PAA – Peracetic acid

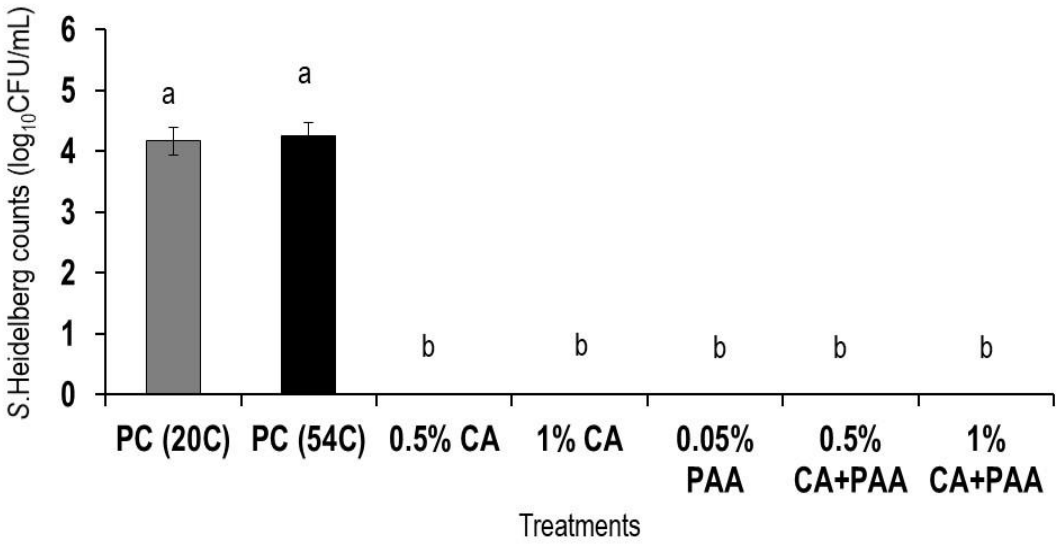


Figure 5A: Effect of caprylic acid against *Salmonella* Heidelberg survival (high inoculum) on chicken drumsticks after 30 minutes of chilling at 4^{o}C (Mean ± SE; n=6). a,b,c – bars with different superscripts are significantly different from each other at $P<0.05$. PC – Positive control, CA – Caprylic acid, PAA – Peracetic acid

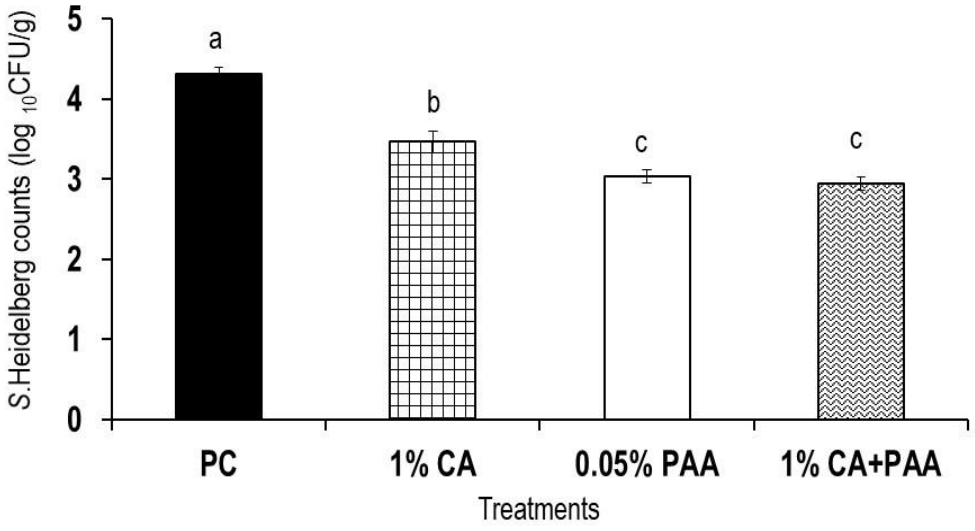


Figure 5B: Effect of caprylic acid against *Salmonella* Heidelberg survival (high inoculum) on chicken drumsticks after 48 hours of storage at 4ºC (Mean ± SE; n=6). a,b,c – bars with different superscripts are significantly different from each other at P<0.05. PC – Positive control, CA – Caprylic acid, PAA – Peracetic acid

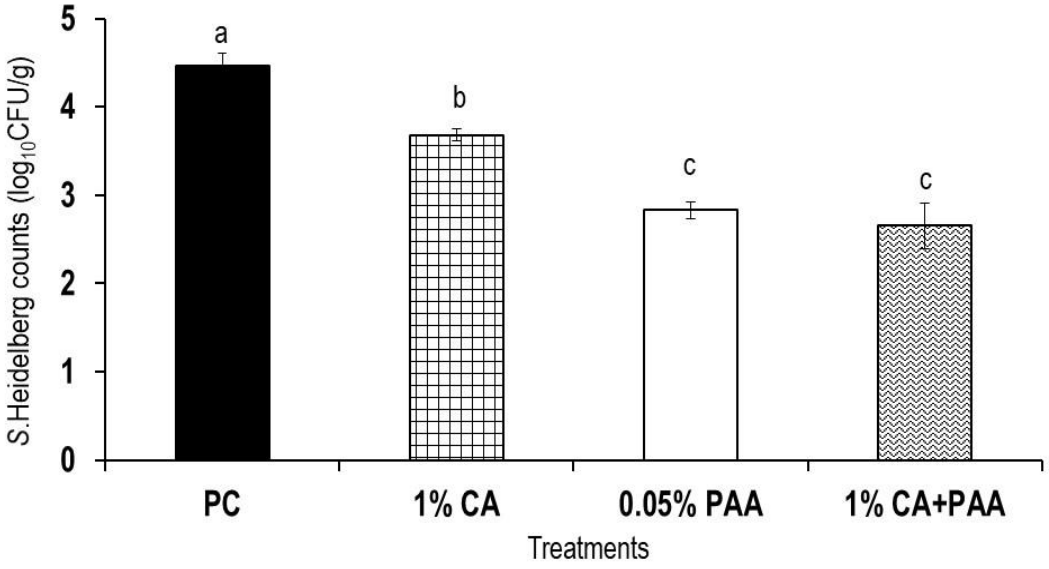


Figure 5c: Effect of caprylic acid against *Salmonella* Heidelberg survival (high inoculum) in chilling water after 30 minutes of chilling at 4^{o}C (Mean ± SE; n=6). a,b,c – bars with different superscripts are significantly different from each other at P<0.05. PC – Positive control, CA – Caprylic acid, PAA – Peracetic acid

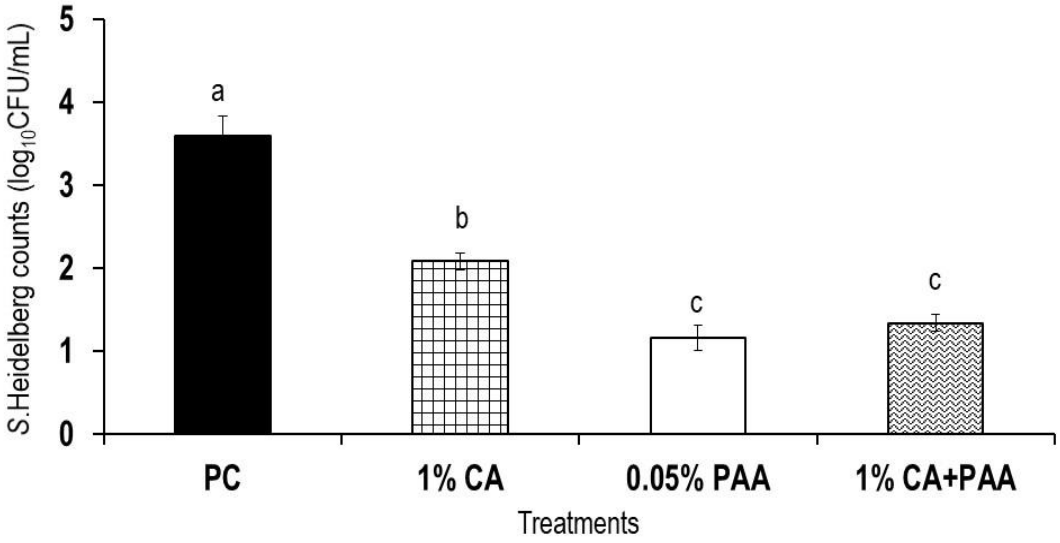


Table 1: Effect of treatments on pH of scalding water.

Treatments	**pH**
NC	6.58±1.06
PC 54℃	6.15±0.93
PC 20℃	6.64±0.75
0.5% CA	4.22±0.05
1% CA	4.19±0.05
PAA	3.39±0.10
0.5% CA+PAA	3.33±0.11
1%CA+PAA	3.29±0.12

Table 2: Effect of caprylic acid on L*, a*, and b* values of the drumstick. Color values of drumsticks (n=6) dipped in different treatment water at 54℃ for 2 minutes (Mean ± SE). [a] – Within a column, treatments with same superscript do not differ significantly (P>0.05); NC – Negative control, CA – Caprylic acid, PAA – Peracetic acid

Treatments	L*	a*	b*
NC	67.4 ± 3.2[a]	0.69 ± 0.59[a]	6.2 ± 0.56[a]
0.5%CA	71.9 ± 2.7[a]	0.75 ± 0.68[a]	7.7 ± 0.78[a]
1%CA	69.5 ± 3.2[a]	0.94 ± 0.97[a]	5.3 ± 0.99[a]
0.05%PAA	70.1 ± 0.8[a]	0.88 ± 0.61[a]	4.5 ± 1.1[a]
0.5%CA+PAA	71.9 ± 1.2[a]	1.00 ± 0.66[a]	6.3 ± 1.5[a]
1%CA+PAA	72.9 ± 1.4[a]	1.00 ± 0.31[a]	4.9 ± 0.93[a]

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